

FORM PTO-1190
(REV. 10-96)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

3123-4000US2

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/284152

INTERNATIONAL APPLICATION NO.
PCT/US97/17669INTERNATIONAL FILING DATE
30 September 1997
(30.09.97)PRIORITY DATE CLAIMED
10 October 1996
(10.10.97)

TITLE OF INVENTION

CHRYSOPORIUM CELLULASE AND METHODS OF USE

APPLICANT(S) FOR DO/EO/US

Mark Aaron EMALFARB, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
 2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
 3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
 4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
 5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
 6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
 7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
 8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
 9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
 10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).
- Items 11. to 16. below concern document(s) or information included:
11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
 12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
 13. ☒ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
 14. ☐ A substitute specification.
 15. ☐ A change of power of attorney and/or address letter.
 16. ☒ Other items or information:
 - First page of the published international application No. WO 98/25633
 - Copy of the International Preliminary Examination Report.
 - Copy of Statement (Declaration) Claiming Small Entity Status under 37 CFR §1.9(c) and §1.27(b) - Individual Assignee

17 <input checked="" type="checkbox"/> The following fees are submitted. BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : Search Report has been prepared by the EPO or JPO \$840.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) \$670.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$760.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$970.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$ 96.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY 840.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	168 - 20 =	148	X\$18.00	\$2,664.00	
Independent claims	23 - 3 =	20	X\$78.00	\$1,560.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$ 260.00	
TOTAL OF ABOVE CALCULATIONS =				\$5,324.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				\$2,662.00	
SUBTOTAL =				\$2,662.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				\$2,662.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$	
TOTAL FEES ENCLOSED =				\$2,662.00	
				Amount to be:	\$
				refunded	
				charged	\$

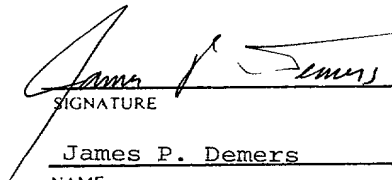
- a. ☒ A check in the amount of \$ 2,662.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees.
 A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
 overpayment to Deposit Account No. 13-4500. A duplicate copy of this sheet is enclosed.

Order No. 3123-4000US2

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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 SIGNATURE
 James P. Demers
 NAME
 34,230
 REGISTRATION NUMBER

Patent

Docket No. 3123-4000US2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (DO/EO/US)

Applicant(s) : Mark Aaron EMALFARB, et al.
International
Application No. : PCT/US97/17669
International
Filing Date : 30 September 1997 (30.09.97)
U.S. Serial No.: : To be Assigned
U.S. Filing Date : Herewith
Group Art Unit: : To be Assigned
Examiner : To be assigned
For : *CHRYSO Sporium* CELLULASE AND METHODS OF USE

Assistant Commissioner for Patents
Box PCT
Washington, DC 20231

Attention: DO/EO/US

PRELIMINARY AMENDMENT

Sir:

With respect to the above-identified international application entering the national phase and filed of even date herewith, please amend this application in the following manner:

In the Specification:

On page 1, after the title, insert:

--RELATED APPLICATIONS

This is a continuation-in-part of US Patent Application Serial No. 08/731,170, filed October 10, 1996, now US Patent 5,811,381.--

66E0990"25T42660

In the Claims:

7. A composition according to any one of claims [2-6] 2 or 4 having cellulase activity at a temperature from about 40⁰C to about 60⁰C, at a pH from about 5.0 to about 11.0.

8. A composition according to any one of claims [2-6] 2 or 4 having at least 50% of the optimal cellulase activity, at a pH from about 6.0 to about 7.0, at a temperature from about 40⁰C to about 60⁰C.

9. A composition according to any one of claims [2-6] 2 or 4 wherein said cellulase activity is assayed by any one of the CMCase, RBBCMCase, endovisco metric or filter paper activity assays.

54. The composition of claim [54] 53, wherein the fungus is *Chrysosporium lucknowense*.

57. The composition of claim [57] 56, wherein the fungus is *Chrysosporium lucknowense*.

83. A mutant strain of the genus *Chrysosporium* obtained by the method of any one of claims [80-82] 80 or 81.

87. An isolated nucleic acid whose sequence encodes a cellulase according to [any one of claims 11-21] claim 11.

88. A recombinant expression vector comprising a nucleic acid sequence according to any one of claims [85-87] 85 or 86.

Add the following new claims:

96. (New) An isolated nucleic acid whose sequence encodes a cellulase according to claim 12.

97. (New) An isolated nucleic acid whose sequence encodes a cellulase according to claim 13.

98. (New) An isolated nucleic whose sequence encodes a cellulase according to claim 14.

99. (New) An isolated nucleic acid whose sequence encodes a cellulase according to claim 15.

100. (New) An isolated nucleic acid whose sequence encodes a cellulase according to claim 19.

101. (New) An isolated nucleic acid whose sequence encodes a cellulase according to claim 20.

102. (New) An isolated nucleic acid whose sequence encodes a cellulase according to claim 21.

103. (New) A recombinant expression vector comprising a nucleic acid sequence according to claim 96.

104. (New) A recombinant expression vector comprising a nucleic acid sequence according to claim 97.

105. (New) A recombinant expression vector comprising a nucleic acid sequence according to claim 98.

106. (New) A recombinant expression vector comprising a nucleic acid sequence according to claim

99.

107. (New) A recombinant expression vector comprising a nucleic acid sequence according to claim 100.

108. (New) A recombinant expression vector comprising a nucleic acid sequence according to claim 101.

REMARKS

The specification has been amended to incorporate a reference to an earlier related application. The claims have been amended to remove improper multiple dependencies. New claims 96-102 substitute for the multiple dependencies of claim 87 which have been removed by amendment. Similarly, new claims 103-108 substitute for the multiple dependencies of claim 88 which have been removed by amendment.

CONCLUSION

The amendments contain no new matter, and entry of the amendments is respectfully requested. Favorable consideration and an action passing this case to issue are therefore respectfully requested. Early and favorable action by the Examiner is earnestly solicited.

If any questions or issues remain, or if the Examiner has any comments or suggestions for expediting allowance of this application, he is urged to contact the undersigned at the telephone number below.

Respectfully submitted,
MORGAN & FINNEGAN, L.L.P.,

Dated: April 8, 1999

By: James P. Demers
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Mark A. Emalfarb *et al.* Group Art Unit : 1652
Serial No. : 08/731,170 Examiner : K. Lau
Filed : October 10, 1996 Issue Batch : F02
For : NOVEL CELLULASE COMPOSITIONS AND METHODS OF USE

**STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS UNDER 37 CFR §1.9(c) AND §1.27 (b) – INDIVIDUAL ASSIGNEE**

As the sole assignee of the entire title and right in the above-referenced patent application, I hereby state that I qualify as an independent inventor as defined in 37 CFR § 1.9(c) and as a small entity as defined in 37 CFR § 1.9(f) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled

NOVEL CELLULASE COMPOSITIONS AND METHODS OF USE

described in application Serial No. 08/731,170 filed October 10, 1996.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR § 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR § 1.9(e).

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (CFR § 1.28(b))

Mark Aaron Emalfarb
Name of Inventor

Signature of Inventor

Date

7-1-98

* NOTE: Separate statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR § 1.27)

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TITLE**CHRYSO Sporium CELLULASE AND METHODS OF USE****FIELD OF THE INVENTION**

5 This invention relates to neutral and/or alkaline cellulases and novel methods for producing the same. More specifically this invention relates to cellulases produced by fungi of the genus *Chrysosporium*, and particular strains of *Chrysosporium lucknowense*. This invention also relates to industrial uses for these neutral or alkaline cellulases and compositions comprising the same.

BACKGROUND OF THE INVENTION

10 Clothing made from cellulosic fabrics such as cotton, linen, hemp, ramie, cupro, lyocell, newcell, rayon, polynosics, are very popular. Of particular interest are clothing items such as jeans made from indigo-dyed denim fabrics made of cotton or cotton blends. Such clothing items are typically sewn from
15 sized and cut cloth and tend to be stiff due to the presence of sizing compositions. In other cases the fibers or rolls of fabric are treated with enzymes prior to sewing the final garment. After a period of wear, the clothing items can develop a certain degree of softness, an overall reduction of shade as well as localized areas of color variation. Additionally, after repeated washing the garment continues to
20 provide a more comfortable fit, a softer feel and a worn appearance. In recent years such comfort, feel and appearance have become increasingly popular.

25 The most widespread methods for producing this comfort, feel and look involve washing of clothing items with cellulases in large washing machines with pumice stones or other abrasives. The pumice helps soften the fabric and helps to provide the faded surface similar to that produced by the extended wear of the fabric. However, the use of pumice has some disadvantages. For example, the pumice must be manually removed from processed clothing items because it tends to accumulate in pockets, on interior surfaces, in creases, and in folds. Also, the
30 pumice stones can cause overload damage to electric motors of stone washing

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machines, and clog machine drainage passages and drain lines. These processing and equipment problems can add significantly to the cost of doing business and to the purchase price of the goods.

In view of the problems of using pumice, alternative methods to using pumice or other abrasives in the stone washing process have been sought. One alternative involves the use of enzyme treatments which break down the cellulose in fabrics (Geller US Patent No. 4,951,366; Olson US Patent's Nos. 4,832,864, 4,912,056, Olson et al. Patent Nos. 5,006,126, 5,122,159 and 5,213,581, Christner et al. U.S. Patent No. 4,943,530, Boegh et al. U.S. Patent No. 4,788,682). Methods for treating cellulose containing fabrics with hydrolytic enzymes, such as cellulases, are known in the art to improve the softness or feel of such fabrics (Novo Brochure Cellulase SP 227; Novo Brochure Celluzyme; Murata US Patent No. 4,443,355; Parslow US Patent No. 4,661,289; Tai US Patent No. 4,479,881; Barbesgaard US Patent No. 4,435,307; Browning UK Patent No. 1,368,599).

Cellulases are known in the art as enzyme systems that hydrolyze cellulose (β -1,4-glucan linkages), thereby resulting in the formation of glucose, cellobiose, celooligosaccharides, and the like. Cellulase compositions are comprised of several different enzyme components, including those identified as exocellobiohydrolases, endoglucanases, and β -glucosidases. Moreover, these classes of enzymes can be further separated into individual isoenzymes.

The complete cellulase system is required to efficiently convert crystalline cellulose to glucose. Generally, if total hydrolysis of a cellulose substrate is needed, the cellulase mixture should contain β -glucosidases and cellobiohydrolases, as well as endoglucanases. Endoglucanases catalyze random hydrolysis of β -1,4-glycosidic bonds between glucose units of cellulose polymers. Such components hydrolyze soluble cellulose derivatives such as carboxymethylcellulose, thereby reducing the viscosity of such solutions. Such enzyme components act on internal regions of the polymer, resulting in a rapid decrease in average polymer chain length together with a slow increase in the

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number of reducing ends. The rapid decrease in average chain length of the cellulose polymer is evidenced by the decrease in viscosity of a cellulose solution.

The substrate specificity and mode of action of the different cellulases varies among strains of organisms that produce cellulases. For example, the currently accepted mechanism of cellulase action in cellulase from the fungus *Trichoderma reesei* is that endoglucanase activity first break internal β -1,4-glucosidic bonds in regions of low crystallinity of the cellulose (Ruohonen L., et al. In: "Proceedings of the Second Tricel Symposium on *Trichoderma Reesei* Cellulases and Other Hydrolases", (ed. by P. Sudminen and T. Reinkainen.,) Foundation for Biotechnology and Industrial Fermentation Research 8; (1993):87-96) The cellobiohydrolase activity binds preferentially to the crystalline regions of the non-reducing end of the cellulose to release cellobiose as the primary product. β -Glucosidase or cellobiase activities then act on celooligosaccharides, e.g., cellobiose, to give glucose as the sole product.

Cellulases are produced in fungi, bacteria, and other microbes. Fungi typically produce a complete cellulase system capable of degrading crystalline forms of cellulose. For example, *Trichoderma reesei* produces and secretes all of the enzyme activities needed for efficient breakdown of crystalline cellulose, namely *endo*-1,4- β -D-glucanases, cellobiohydrolases (*exo*-1,4- β -D-glucanases), and 1,4- β -D-glucanases, or β -glucosidases. Fungal cellulases have an added advantage in that cellulases in fungi can readily be produced in large quantities via fermentation procedures.

Cellulases, or the components thereof, are known in the art to be useful in a variety of industrial textile applications in addition to the stone washing process. For example, cellulases are used in detergent compositions, for the purpose of enhancing the cleaning ability of the composition, as a softening agent, for color brightening, depilling and other uses. When so used, the cellulase will degrade a portion of the cellulosic material, e.g., cotton fabric, in the wash, which facilitates the cleaning and/or softening of the fabric. The endoglucanase components of fungal cellulases have also been used for the purposes of enhancing

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the cleaning ability of detergent compositions, for use as a softening agent, and for use in improving the feel of cotton fabrics, and the like. However, there is a problem with using the cellulase derived from *Trichoderma* spp. and especially *Trichoderma longibrachiatum* in detergent compositions. Generally, such components have their highest activity at acid pHs whereas most laundry detergent compositions are formulated for use at neutral or alkaline conditions.

Other textile applications in which cellulases have been used include softening (Browning, UK Patent No. 1,368,599, Parslow, U.S. Patent No. 4,661,289, Tai U.S. Patent No. 4,479,881 and Barbesgaard, U.S. Patent No. 4,435,307), defibrillation (Gintis, D. Mead, E.J., Textile Research Journal, 29, 1959; Cooke, W.D., Journal Of The Textile Research Institute, 74, 3, 1983; Boegh, European Patent Application No. 0 220 016). Cellulases have also been used in combination with a polymeric agent in a process for providing localized variation in the color density of fibers. (WO/94/19528 and WP/94/1529).

Cellulases are classified in the garment and textile industry according to their pH range of operation. Acid cellulases typically have their peak activity at pH values of about 4.0 to 5.5 and less, neutral cellulases at about pH 5.5 to 7.5, and alkaline cellulases at about pH 7.5 to 11.0. Some enzyme compositions may have broader ranges of operation. For example, the neutral/alkaline cellulases may operate at acid, neutral and alkaline pH's at between about 40°C to 60°C.

Acid, neutral and alkaline cellulases are typically used in the "stone wash" treatment of denim jeans, with or without surfactants, buffers, detergents, anti-redeposition agents, softening agents, pumice stones or other abrasives, bleaching agents, such as optical bleaching agents, enzymes, or other means. If the cellulase composition is not formulated and/or pre-buffered then for acid cellulases, the pH is typically adjusted to between pH 4.5 - 5.5, with for example, a sodium citrate and citric acid buffer, and for neutral or alkaline cellulases between 5.5 - 7.5 with, for example, a monosodium and disodium phosphate buffer. Neutral and alkaline cellulases are typically used as additives to laundry

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detergents where the pH of operation may range from about pH 7.0 to 11.5. In stone wash applications typical acid cellulases generally provide greater backstaining or redeposition of the indigo dye and greater strength loss of the fabric while the typical neutral and alkaline cellulases generally provide less abrasion, lower backstaining or redeposition and less strength loss of the fabric.

The neutral/alkaline cellulases are the most preferred type of cellulases for the stonewash industry because they cause lower levels of backstaining or redeposition and lower strength loss than acid cellulases (*ie*; from *Trichoderma* sp.). Furthermore, neutral/alkaline cellulases, unlike their acid counterparts, operate at a much wider pH range and are able to maintain better relative wash performance within a wider pH range (pH 5.0 - pH 8.0) in the stone washing industry. Therefore, neutral/alkaline cellulases provide several advantages. First, the incoming feed water in wet processing facilities is typically within this pH range lessening the need for as precise pH control as compared to acid cellulases. This makes the stonewashing process more tolerant to operator pH error or neglect leaving the overall procedure more forgiving than procedures using acid cellulases. Secondly, it is known that denim fabrics are alkaline in nature owing to the fact that the dyeing process utilizes caustic soda. Simply washing denim releases this caustic into the wash water and the pH of the wash water generally rises. The alkalinity may overcome the bath buffers, but the effect of increased pH is less severe on neutral/alkaline cellulases compared to acid cellulases because neutral/alkaline cellulases operate not only at higher pH, but also over a wider pH range.

The wide spectrum of industrial uses for cellulases or the components of cellulases, especially alkaline and/or neutral cellulases, establishes a clear need for cellulases that are operative at neutral and/or alkaline pH. The present invention provides a procedure for producing neutral/alkaline cellulases having enzymatic activity at neutral and/or alkaline pH's and compositions comprising the same.

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SUMMARY OF THE INVENTION

This invention relates, in general, to neutral and/or alkaline cellulases and novel methods for producing the same. More specifically, the subject invention provides a method for producing cellulase compositions from fungi of the genus *Chrysosporium*, and particular *Chrysosporium lucknowense*, wherein the cellulase compositions have enzymatic activity at neutral and/or alkaline pH's. Industrial applications for the cellulase composition are also provided.

One embodiment of this invention relates to isolated and purified cultures of wild type and mutant fungi of the genus *Chrysosporium* capable of producing neutral and/or alkaline cellulase compositions, in particular to the strain *Chrysosporium lucknowense* - GARG 27K and mutants thereof.

Yet another embodiment of this invention provides culturing conditions for producing neutral or alkaline cellulases from fungi of the genus *Chrysosporium*.

In a further embodiment, this invention provides methods to producing a neutral and/or alkaline cellulase composition through recombinant technology from fungi of the genus *Chrysosporium*.

In yet a further embodiment of this invention methods for generating and culturing mutant strains of the fungi *Chrysosporium* capable of producing neutral and/or alkaline cellulase are provided.

Another embodiment of this invention relates to the nucleic acid sequences encoding the enzymes of the cellulases compositions produced by *Chrysosporium* or genetically modified strains of *Chrysosporium*.

Another embodiment relates to the purified and isolated enzymes of the cellulase compositions produced by *Chrysosporium* or genetically modified strains of *Chrysosporium*.

In yet another embodiment of this invention methods of use are provided for alkaline and/or neutral cellulases produced by *Chrysosporium* in textile applications, such as softening, bleaching and stone washing procedures,

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garment dyeing applications, defibrillation, or biopolishing, color brightening and depilling.

Another embodiment of this invention relates to detergent compositions comprising *Chrysosporium* cellulase in detergent preparations.

5 Another embodiment of this invention is to provide methods of use for the cellulase compositions in the saccharification of lignocellulose biomass from agriculture, forest products, municipal solid waste, and other sources.

Yet other embodiments of this invention involve the use of the cellulase compositions for production of fuels and other chemicals for the biobleaching of wood pulp, and for de-inking of recycled print paper.

Detailed Disclosure of the Invention

As utilized herein, reference to a "neutral-alkaline cellulase" refers to a cellulase composition which retains significant enzymatic activity at pH values of about 5.5 and above. In a preferred embodiment, the neutral and/or alkaline cellulase compositions of the subject invention have peak enzymatic activity between about pH 5.5 to about 7.5 at 40°C to about 60°C. In the event that the peak enzymatic activity is at a pH of less than about 5.5, the neutral-alkaline cellulase composition will have at least about 50% of the optimal enzymatic activity at about pH 6.0 to about 7.0 at about 40°C to about 60°C. By way of example such activities may be measured by RBBCMCase, CMCase, Cellazyme, endoviscometric or filter paper activity (FPA). Thus, the cellulase compositions of the subject invention will have useful enzymatic activity at pHs greater than 5.5 such that the enzyme composition can be used in stone wash, detergent, de-inking or other applications where neutral and/or alkaline cellulase activity is needed.

The subject invention relates to compositions of cellulases having high activity at neutral or alkaline pH's and to unique methods for producing said neutral and alkaline cellulase compositions. The neutral/alkaline cellulase compositions of this invention may be obtained from any species of *Chrysosporium*. In a particularly preferred embodiment, the cellulase

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compositions of the present invention are isolated from *Chrysosporium lucknowense* Garg 27K (designated isolate C1) deposited under the Budapest Treaty with the International Depository at the All-Russian Collection of Microorganisms of the Russian Academy of Sciences, Bakhrushina St. 8: Moscow, Russia 113184, on August 29, 1996, and assigned accession number VKM F-3500D. The cellulase compositions of the subject invention are highly advantageous because they possess enzymatic activity at neutral and/or alkaline pH thereby providing beneficial performance characteristics in industrial applications.

The cellulase compositions prepared from fungal strains of the subject invention exhibit activity at between about pH 5.0 to about 12.0 at between about 40° to 60°C as determined by a CMCase, RBBCMCase, Cellazyme, endoviscometric or Filter Paper Activity (FPA) assays. In a preferred embodiment for a stone wash procedure, the cellulase composition may have optimal activity at between about pH 5.5 to 7.0 at about 40°C to about 60°C. Good performance activity at neutral and alkaline pH (ie: 6.0, 7.0 & 8.0) has been demonstrated for the neutral and/or alkaline cellulases of the instant invention in Stonewash application trials and at pH 10.0 and above for detergent application trials.

The fermentation procedures for culturing cellulolytic microorganisms for production of cellulase are known in the art. For example, cellulase systems can be produced either by solid or submerged culture, including solid state, batch, fed-batch, and continuous-flow processes. The collection and purification of the cellulase systems from the fermentation broth can also be effected by procedures known in the art. The cellulase composition is readily isolated from the fungal culture by, for example, centrifugation or filtration steps and concentration of the filtrate via membrane or hollow fibers ultrafiltration equipment.

The fungal strain *Chrysosporium* used to produce the cellulase compositions of the subject invention can be cultured according to standard methods and conditions known in the art. In a preferred embodiment, the cellulase composition of the subject invention is obtained from the C1 strain. The C1

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Chrysosporium strain may be grown in a medium containing inorganic salts, organic nitrogen sources, such as peptones, defatted cotton seed flour, corn steep liquor, or yeast extract and carbon source. Examples of carbon source include, but is not limited to, glucose, lactose, sucrose, cellulose or other carbohydrates. More preferably, the fungal strain is grown in media containing both lactose and peptone or lactose and yeast extract. By way of example the fermentation media can compose lactose at about 0.3% to about 1.0%, preferably about 0.5% to about 0.6%, peptone at about 0.3% to about 1.0%, preferably about 0.5% to about 0.6%. Other nitrogen sources and carbohydrate sources known in the art may be used in the fungal growth media including, but not limited to, sweet beet pulp, barley malt, wheat bran, and others known in the art. By way of example sweet beet pulp concentrate may be used in a range of about 15 to about 30 grams/liter (g/L), preferably about 20 to about 25 g/L; barley malt may be used in a range about 10 g/L to about 20g/L, preferably about 14 g/L or about 16g/L, wheat bean may be used in a range about 3g/L to about 8g/L, preferably about 5g/L to about 6 g/L. In one embodiment, the C1 strain is cultured in rotated shake flasks in saline medium containing sweet beet pulp, barley malt, and wheat bran. Cellulase compositions may be isolated from fungi cultured about 3 to 7 days in a growth medium by centrifugation and ultrafiltration concentration of the cell culture medium.

Alternatively the *Chrysosporium* cultures can be cultured on a large scale for commercial use, by using conventional fermentation techniques. In this context fermentation is used broadly to refer to any controlled fungal culturing conditions. Prior to large scale growth an inoculum of said growth culture is generally cultured. The inoculum media may contain conventional ingredients including, but not limited to, carbon sources, organic nitrogen sources, and inorganic salts. Carbon sources may include, but are not limited to, glucose, lactose, glycerol, and/or cellulose at concentrations in the range of about 0.5 to 200 g/L, more preferably in the range of about 5 to 50 g/L. Organic nitrogen sources may include, but are not limited to, yeast extract, peptone, or defatted

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cotton seed flour at concentrations in the range of about 0.5 to 30 g/L, more preferably in the range of 5 to 15 g/L. Inorganic salts may include, but are not limited to, potassium phosphate, for example at about 0.01 to about 10 g/L, magnesium sulfate, for example at about 0.01 to 3.0 g/L, ferrous sulfate, for example at about 0.001 to 10 mg/L.

An inoculum or starter culture may be used to initiate the *Chrysosporium* culture for a fermenter by methods known in the art. The media used for fermentation may comprise conventional ingredients for culturing fungi, including but not limited to, cellulose, organic nitrogen sources, magnesium chloride and calcium chloride. Examples of organic nitrogen sources include, but are not limited to, peptone or defatted cotton seed flour, such as Pharmamedia.

By way of example, the media may comprise about 5g/l to about 20 g/L of peptone or defatted cotton seed flour, about 10g/L to about 30g/L of cellulose, about 0.03 g/L to about 0.06 g/L of magnesium sulfate heptahydrate and about 0.4 g/L to about 0.8 g/L of calcium chloride dihydrate.

One of skill in the art will appreciate that during fermentation the temperature, oxygenation, pH, and nutrient levels of fermentation mixture should be maintained. By way of example, dissolved oxygen levels should be maintained at about 10 to 60% of air saturation, preferably at about 20 to 40% of air saturation. The pH should be maintained between about 5 and 8, preferably between about 6.5 and 7.5, most preferably between 6.9 and 7.1 and the temperature may be maintained at between about 25°C to about 40°C, preferably at about 28°C to 35°C. The feed solution may comprise ingredients similar to the fermentation media but at higher concentrations to minimize dilution when added to the fermentation media.

The cellulase compositions produced according to the methods of the subject invention are useful for a variety of other applications for which cellulase activity, in particular neutral and/or alkaline cellulase activity, is needed. In one embodiment of this invention, the neutral and/or alkaline cellulase compositions can be used in stone washing procedures for denim jeans. By way of example, the

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most preferred pH range of stone wash applications is between about 5.5 to 7.5, most preferably at about pH 6 to about 7. The neutral and/or alkaline cellulase composition obtained from *Chrysosporium* isolates advantageously have significant enzymatic activity at or above neutral or alkaline pH. Stone wash procedures conducted with neutral and/or alkaline cellulase run at neutral and/or alkaline pH's are particularly advantageous compared to traditional procedures using acid cellulases (eg: those from *Trichoderma reesei*) because of lower levels of backstaining on the garments, less strength loss to the garments and the alkalinity of the water that is present naturally during this process. These stone washing procedures result in jeans with highly desirable feel and appearance. By way of example, 0.02 to 10g of cellulase preparation 47.0528 described herein, may be used per 135g of denim. One of skill in the art will know how to regulate the amount or concentration of the cellulase composition produced by this invention based on such factors as the activity of the cellulase, and the wash conditions, including but not limited to temperature and pH.

In yet another embodiment of this invention, the cellulase compositions of this invention can be used to reduce or eliminate the harshness associated with fabrics made from cellulose by addition to detergent compositions. By way of example, the preferred range for detergent compositions is between about pH 8 to about 12, most preferably pH 10 to about 11. The cellulase compositions of the subject invention can be used in detergent compositions at neutral and or alkaline pH. Detergent ingredients contemplated for use with the cellulase composition of the subject invention include any detergent ingredient known in the art. Examples of such ingredients include, but are not limited to, detergents, buffers, surfactants, bleaching agents, softeners, solvents, solid forming agents, abrasives, alkalis, inorganic electrolytes, cellulase activators, antioxidants, builders, silicates, preservatives, and stabilizers, and are known in the art. The detergent compositions of this invention preferably employ a surface active agent, *i.e.*, surfactant, including anionic, non-ionic, and ampholytic surfactants well known for their use in detergent compositions. In addition to the

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cellulase components and the surface active agent, the detergent compositions of this invention can additionally contain one or more of the following components; the enzymes amylases, cellulases, proteinase, lipases, oxido-reductases, peroxidases and other enzymes; cationic surfactants and long-chain fatty acids; builders; antiredeposition agents; bleaching agents; bluing agents and fluorescent dyes; caking inhibitors; masking agents for factors inhibiting the cellulase activity; cellulase activators; antioxidants; and solubilizers. In addition, perfumes, preservatives, dyes, and the like can be used, if desired, with the detergent compositions of this invention. Examples of detergent compositions employing cellulases are exemplified in Patent No's 4,435,307; 4,443,355; 4,661,289; 4,479,881; 5,120,463, which are herein incorporated by reference.

When a detergent base used in the present invention is in the form of a powder, it may be one which is prepared by any known preparation method including a spray-drying method and/or a granulation method. The granulation method are the most preferred because of the non-dusting nature of granules compared to spray dry products. The detergent base obtained by the spray-drying method is hollow granules which are obtained by spraying an aqueous slurry of heat-resistant ingredients, such as surface active agents and builders, into a hot space. The granules have a size of from about 50 to about 2000 micrometers. After the spray-drying, perfumes, enzymes, bleaching agents, and/or inorganic alkaline builders may be added. With a highly dense, granular detergent base obtained by such as the spray-drying-granulation method, various ingredients may also be added after the preparation of the base. When the detergent base is a liquid, it may be either a homogenous solution or an inhomogeneous solution.

The cellulase compositions of this invention preferably exhibit high levels of activity at alkaline or neutral pH's, but also may exhibit enzymatic activity at acidic pH's. Therefore, the detergent compositions comprising the cellulases of the present invention can be used in a broad pH range of from acidic to alkaline pH.

Other textile applications in which these cellulase compositions may

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be used include, but are not limited to, Garment Dyeing applications including but not limited to Enzymatic Mercerizing of viscose, Bio-Polishing applications, Enzymatic Surface Polishing; Biowash (washing or washing down treatment of textile materials), Enzymatic Microfibrillation, Enzymatic "cottonization" of linen, ramie and hemp; and treatment of Lyocel or Newcell (ie; "TENCEL" from Courtauld's), Cupro and other cellulosic fibers or garments, dye removal from dyed cellulosic substrates such as dyed cotton (Leisola & Linko - (1976) Analytical Biochemistry, v. 70, p. 592. *Determination Of The Solubilizing Activity Of A Cellulase Complex With Dyed Substrates*; Blum & Stahl - Enzymic Degradation Of Cellulose Fibers; Reports of the Shizuoka Prefectural Hamamatsu Textile Industrial Research Institute No. 24 (1985)), as a bleaching agent to make new indigo dyed denim look old (Fujikawa - Japanese Patent Application Kokai No. 50-132269), to enhance the bleaching action of bleaching agents (Suzuki - Great Britain Patent No. 2 094 826), and in a process for compositions for enzymatic desizing and bleaching of textiles (Windbichtler et al., U.S. Patent No. 2,974,001. Another example of enzymatic desizing using cellulases is provided in Bhatawadekar (May 1983) Journal of the Textile Association, pages 83-86.

In other industrial embodiments, the cellulase compositions can be used in the saccharification of lignocellulose biomass from agriculture, forest products, municipal solid waste, and other sources, for the production of fuels and other chemicals through fermentation, for biobleaching of wood pulp, and for de-inking of recycled print paper all by methods known to one skilled in the art.

In yet another embodiment of the subject invention, various components of the neutral and alkaline cellulase compositions can be isolated and used independently of each other. Specific components or cellulase composition enriched by certain cellulase components can be produced or isolated by chemical and physical means from mutants or specifically produced by genetic engineering methods. The cellulase system can be purified into separate components by art-recognized separation techniques including ion exchange chromatography at a suitable pH, affinity chromatography, size exclusion, chromatography and like.

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For example, in ion exchange chromatography, it is possible to separate the cellulase components by eluting with a pH gradient, or a salt gradient, or both. Such separations can be done by those skilled in the art having the benefit of the teachings provided herein.

5 Once the individual enzymatic components of the cellulase composition are fractionalized and isolated the proteins may be partially sequenced or microsequenced to design synthetic DNA or probes to isolate the gene encoding the enzymatic proteins of interest. Generally the amino terminal sequence of the protein is determined by conventional protein sequencing methods or by automated
10 sequence (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York). Alternatively, other regions of the protein may be sequenced in combination with chemical cleavage or enzymatic cleavage and protein separation techniques. (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York).
15 One of skill in the art will understand that the synthetic DNA clones or probes can be used in routine cloning techniques to isolate the genes corresponding to the enzymes present in the neutral/alkaline cellulase compositions produced by *Chrysosporium*.

20 It will be understood by one skilled in the art that nucleic acid sequences obtained by this invention in the art may vary due to the degeneracy of the genetic code variations in the DNA sequence, but will still result in a DNA sequence capable of encoding the enzymatic components of the cellulase compositions. Such DNA sequences are therefore functionally equivalent to the nucleic acid sequences of the instant invention and are intended to be encompassed
25 within the present invention. Also intended to be encompassed within this invention are nucleic acid sequences which are complementary to nucleic acid sequences capable of hybridizing to the disclosed nucleic acid sequence under a variety of conditions.

30 This invention further includes the nucleic acid sequences encoding the enzymes of the cellulase compositions of this invention and

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those proteins or peptides having substantially the same function as the enzymatic proteins or peptides of this invention. Such proteins or polypeptides include, but are not limited to, a fragment of the protein, or a substitution, addition or deletion mutant. This invention also encompasses proteins or peptides that are substantially homologous to the proteins encoding the enzymes comprising the cellulase composition of this invention. The term "analog" includes any polypeptide having an amino acid residue sequence substantially identical to the sequence specifically in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of the proteins as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or alanine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between threonine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

Proteins or polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions or residues relative to the sequence of a polypeptide whose sequence is included in the proteins of this invention so long as the requisite activity is maintained.

This invention also provides a recombinant DNA molecule comprising all or part of the nucleic acid sequences isolated by this invention and a vector. Expression vectors suitable for use in the present invention comprise at least one expression control element operationally linked to the nucleic acid sequence. The expression control elements are inserted in the vector to control and regulate the expression of the nucleic acid sequence. Examples of expression control elements include, but are not limited to, lac system, operator and promoter regions of phage lambda, yeast or other fungi promoters. Examples of promoters that may be used include, but are not limited to, glucoamylase. Additional preferred or required operational elements include, but are not limited to, leader

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sequence, termination codons, polyadenylation signals and any other sequences necessary or preferred for the appropriate transcription and subsequent translation of the nucleic acid sequence in the host system. It will be understood by one skilled in the art the correct combination of required or preferred expression control elements will depend on the host system chosen. It will further be understood that the expression vector should contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication and selectable markers. It will further be understood by one skilled in the art that such vectors are constructed using conventional methods (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York) or may be commercially available.

Another aspect of this invention relates to a host organism into which recombinant expression vector containing all or part of the nucleic acid sequence has been inserted. The host cells transformed with the nucleic acid sequence of this invention includes eukaryotes, such as animal, plants or seeds, insect and yeast cells, fungal cells, and prokaryotes, such as *E. coli* or other bacteria. Examples of fungal host cells include but are not limited to *Aspergillus*, *Trichoderma*, *Humicola*, *Penicillium*, or *Neurospora*. The means by which the vector carrying the gene may be introduced into the cell include, but are not limited to, transformation, microinjection, electroporation, transduction, or transfection using DEAE-dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art (Sambrook et al. (1989) in "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Press, Plainview, New York). Alternatively, *Chrysosporium* cells can be transformed with the nucleic acid sequence of this invention to amplify production of cellulases by *Chrysosporium*.

In a preferred embodiment, expression vectors that function in fungal cells are used. Examples of such vectors include, but are not limited to

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plasmids, described in the patents (Ogawa; Japanese patent JP5095787 A 930420, Ozeki; Japanese patent JP7115976 A 950509, Murakami; Japanese patent JP3094690 A 910419, Ishida; Japanese patent JP3251175 A 911108, Uozumi; Japanese patent JP5268953 A 931019 DW9346 C12N-009/34 011pp, Gottschalk; German patent DE3908813 A 900920 DW9039 000 pp, Gysler; European patent EP-683228 A2 951122 DW9551 C12n-015/60 Eng 041 pp). It is preferred that the recombinant protein expression vector is introduced into fungal cells, such to ensure proper processing and modification and modification of the introduced protein.

In a further embodiment, the recombinant protein expressed by the host cells can be obtained as a crude lysate or can be purified by standard protein purification procedures known in the art which may include differential precipitation, molecular sieve chromatography, ion-exchange chromatography, isoelectric focusing, gel electrophoresis, affinity, and immunoaffinity chromatography and the like. (Ausubel et. al., (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York). In the case of immunoaffinity chromatography, the recombinant protein may be purified by passage through a column containing a resin which has bound thereto antibodies specific for the protein of interest (Ausubel et. al., (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York).

All or parts of the nucleic acid sequences of this invention can also be used as probes to isolate other homologs in other genera or strains. In a preferred embodiment the nucleic acid sequences are used to screen a *Chrysosporium* library; positive clones are selected and sequenced. Examples of sources from which the gene library can be synthesized include, but are not limited to species of *Chrysosporium*, *Aspergillus*, *Penicillium*, *Humicola*, *Cephalosporium*, *Trichoderma* or bacteria such as *Bacillus*. One skilled in the art will understand the appropriate hybridization conditions to be used to detect the homologs. Conventional methods for nucleic acid hybridization, construction of libraries and cloning techniques are described in Sambrook et al., (eds) (1989) In "Molecular

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Cloning A Laboratory Manual" Cold Spring Harbor Press, Plainview, New York and Ausubel et al., (eds) in "Current Protocols in Molecular Biology" (1987), John Wiley and Sons, New York, New York.

This invention also relates to mutant strains of *Chrysosporium*, in particular, mutant strains of *Chrysosporium lucknowense* capable of producing neutral and/or alkaline cellulases. Methods of DNA mutagenesis and screening for mutants are well known to those skilled in the art and include a variety of chemical and enzymatic methods. Examples of such methods include but are not limited to exposure of the fungi to ultraviolet light (UV), nitrous acid, N-methyl-N'-nitro-N-nitrosoguanidine (NG), and 4-nitroquinoline-N-oxide (4NQO). (Leninger (1972) Biochemistry, Worth Publishers Inc., NY; Jeffrey H. Miller (1972) "Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Springs Harbor, New York). Preferred methods, include UV and NG. By way of example, mutant strains of *Chrysosporium* capable of producing increased levels cellulase compositions exhibiting enzymatic activity at neutral and or alkaline pH's may be generated by UV mutagenesis. By way of examples mutants may be produced by exposure of the fungal spores to UV light for a period of about 10 to about 180 seconds, preferably 45 to about 90 seconds, and most preferably 65 to 75 seconds. Mutageneses involving NG may involve varying the concentration of the NG and exposure time. By way of example NG, at about 13 milligrams/liter (mg/L) to about 400mg/L of NG may be used for a period of exposure of about 15 minutes to about 120 minutes. Alternative methods for generating mutants include techniques in the field of molecular biology. Examples of such techniques include, but are not limited to, oligonucleotide directed mutagenesis, linker scanning mutations or oligonucleotide directed mutagenesis using polymerase chain reaction (Ausubel (1987) Current Protocols in Molecular Biology).

Screening and selection of mutant *Chrysosporium* clones exhibiting improved cellulase production can also be performed by conventional methodology. Examples of selection criteria for screening mutants includes, but is not limited, to the ability of the fungal colony to breakdown cellulase as evidenced

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by cellulose clearing by the fungi grown on media plates containing cellulose and increased growth rate on media containing cellulose. By way of example, after mutagenesis the fungal sample may be plated on agar plates containing cellulose by conventional methodology. Colonies exhibiting increased growth and cellulose clearing (as is evidenced by clearing of the media surrounding the isolate) may be selected. However, any other assay for cellulose clearing may be used. Once a mutant is isolated the stock or spores derived from the mutant may be maintained by conventional methodology. The mutants isolated by this method may be cultured or fermented under the conditions described herein and above for the wild type strains of *Chrysosporium*. The individual enzymatic components of the cellulase composition produced by the mutants may be partially sequenced or microsequenced to design synthetic to isolate the genes encoding the enzymatic proteins of interest. Thus, this invention relates to the nucleic acid sequences encoding the cellulase enzymes produced by the *Chrysosporium* mutants and to the enzymes themselves. In yet another embodiment of this invention, the mutants isolated may be subjected to further mutagenesis and screened by the methods described herein above.

The present invention also relates to the production, isolation and purification of cellulase enzymes, having both endoglucanase and/or cellobiohydrolase activity from *Chrysosporium* organisms of any species and strain desired and wherein the purification methodology is not limited to the species of organism. Such purified, or partially purified, protein fractions and enzymes prepared therefrom are, in accordance with the present invention, highly useful in applications such as stone washing, color brightening, depilling and fabric softening, as well as other applications well known in the art. Such purified enzyme preparations are readily amenable to use as additives in detergent and other media used for such applications. These and other methods of use will readily suggest themselves to those of skill in the art and need no detailed description herein. However, description of typical methods of employing these preparations is provided in the examples that follow.

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All books, patents or articles references herein are incorporated by reference. The following examples illustrate various aspects of the invention but in no way are included to limit the invention. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

5

Materials and Methods

Enzyme assays. The assay for CMCase, used carboxymethyl cellulose as the enzymatic substrate, measured the initial rate of hydrolysis, and quantified the amount of reducing sugars released according to the method of Somogyi and Nelson. The method is described in *Methods in Enzymology*, Vol. 160A, pp. 102-104. Endo-1,4- β -glucanase activity was assayed viscometrically as a rate of the decrease of viscosity of soluble substrate CMC according to *Bioorganicheskaya Khimia*, Vol. 6, pp. 1225-1242 (endoviscometric assay). Filter paper activity (FPA) or total cellulase activity used filter paper as the substrate and estimated the activity required to release 2 mg glucose from a 50-mg sample of filter paper. The assay is based on the Commission on Biotechnology (IUPAC) for the measurement for total cellulase activity or true cellulase and is described in *Methods in Enzymology*, Vol. 160A, pp. 94-97. Avicelase activity was estimated as initial rate of reducing sugar formation during hydrolysis of Avicell-cellulose (as described in *Bioresource Technology*, Vol. 52, pp. 119-124). The assay of cellobiase used cellobiose as the substrate and measured the amount of glucose released (described in *Methods in Enzymology*, Vol. 160A, pp. 111-112). The assay of β -glucosidase activity used *p*-nitrophenyl- β -D-glucoside as the substrate (described in *Methods in Enzymology*, Vol. 160A, pp. 109-1100). The protein was determined by Lowry method (according to *Journal of Biological Chemistry*, Vol. 193, pp. 165-175). The assay of RBB-CMCase is based on determination of the dye release from soluble substrate RBB-CMC (CMC, dyed with Remazolbrilliant Blue), reference to assay - see Megazyme (Australia) Ltd., Product Information Bulletin, April, 1995. Endo-cellulase can also be measured using the Cellazyme assay with Azurine-crosslinked HE-cellulose as substrate (see Megazyme Product

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Bulletin CEL, January, 1996).

Example 1 -- Isolation of C1 Strain

The strain was isolated from samples of forest alkaline soil from Sola Lake, Far East of Russian Federation (Pacific Coast of Russia, about 5000 miles east from Moscow). A mixed soil sample was collected from 10 different sites. One gram of each sample was transferred into a flask with 100 ml sterile tap water and sonicated with an ultrasonic dispenser for 1 minute (0.44 Amp, 22 KHz). The suspension (diluted 1:500) was inoculated into petri dishes with Czapek medium (pH 5.5-6.0) containing 100 mg/L streptomycin. The study was conducted in three replicates. Colonies of various color shape and size were identified for a second isolation step. Further isolation of the sample was performed on plates with Czapek media, malt agar, potato dextrose agar, or Getchinson saline medium pH 7.5 (Table 2). Plates were incubated at about 28°C for several days. Selection for cellulase producers was performed on cellulose agar plates which contained the components shown in Table 1. Preparation of amorphous cellulose is described in *Methods in Enzymology* vol. 160A.

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Table 1. Cellulose agar plates

Ingredients	g/L
KH ₂ PO ₄	1
KCl	0.1
MgSO ₄ ·7H ₂ O	0.3
NaCl	0.1
FeCl ₃	0.01
NaNO ₃	2.5
Amorphous cellulose	5
Agar	15
pH	7.5

The plates were incubated for 3-7 days at 28°C. The formation of light clearing halos around the colonies indicated cellulase activity. One strain, designated herein as C1, that exhibited significant levels of cellulase activity was chosen for additional study. The strain was deposited at the All-Russian Collection of Microorganisms of Russian Academy of Sciences, (VKM), abbreviation in English - RCM), Bakhrushina St. 8: Moscow, Russia, 113184 under the Budapest Treat on August 29, 1996, as *Chrysosporium lucknowense* Garg 27K, VKM-F 3500 D).

Example 2 -- Characterization of C1 Strain

Growth of the C1 strain on potato dextrose agar gives colonies of 55-60 mm diameter after 7 days. C1 colonies exhibit a white-cream color, the surface is velvet-like and has a slightly raised center. The edge of the colonies is a flat, thin and fibereil. The back side of the colonies has a light cream color.

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The mycelium has hyaline and is slightly branched and smooth. The hyphae are thin-walled. Air hyphae are septate and form spores of 2.0-3.0 micrometers width; the substrate hyphae are sterile.

The conidia are terminal and lateral. No intercalary conidia were found. The majority of conidia are connected with hyphae through short stems or short side branches. The conidia are separated but adjacent. Conidia are hyaline, thin-walled, oval or clavate, and single cellular. Their size varies from 4 to 10 micrometers in diameter.

The C1 strain can be maintained on malt extract agar (at 4°C), and transferred each six months. Maintenance in liquid nitrogen and by lyophilization is also possible. The C1 strain is haploid, filamentous, can grow on agar plates with growth restricting agents like bovine bile (1.5 %), and produces spores.

Example 3 -- Classification of C1 Strain

According to Sutton classification (Van Dorschot, C.A.N. [1980] "A revision of *Chrysosporium* and allied genera," in *Studies in Mycology*, No. 20, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, pp. 1-36), the C1 strain of the subject invention belongs to the order of *Hyphomycetales*, family of *Moniliaceae*, genus of *Chrysosporium*, species of *Chrysosporium lucknowense* Garg 1966. This classification was based on observation of the following characteristics of the C1 strain:

1. Signs of *Hyphomycetales* order. Conidia are produced directly on mycelium, on separate sporogenous cells or on distinct conidiophores.

2. Signs of *Moniliaceae* family. Both conidia and conidiophores (if present) are hyaline or brightly colored; conidiophores are single or in loose clusters.

3. Signs of *Chrysosporium* Corda 1833 genus. Colonies are usually spreading, white, sometimes cream-colored, pale brown or yellow, felty and/or powdery. Hyphae are mostly hyaline and smooth-walled, with irregular, more or less orthotopic branching. Fertile hyphae exhibit little or no differentiation.

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Conidia are terminal and lateral, thallic, borne all over the hyphae, sessile or on short protrusions or side branches, subhyaline or pale yellow, thin- or thick-walled, subglobose, clavate, pyriform, orobovoid, 1-celled, rarely 2-celled, truncate. Intercalary conidia are sometimes present, are solitary, occasionally catenate, subhyaline or pale yellow, broader than the supporting hyphae, normally 1-celled, truncate at both ends. Chlamydospores are occasionally present.

4. Signs of *Chrysosporium lucknowense* Garg 1966 species.

Colonies attain 55 mm diameter on Sabouraud glucose agar in 14 days, are cream-colored, felty and fluffy; dense and 3-5 mm high; margins are defined, regular, and fimbriate; reverse pale yellow to cream-colored. Hyphae are hyaline, smooth- and thin-walled, little branched. Aerial hyphae are mostly fertile and closely septate, about 1-3.5 mm wide. Submerged hyphae are infertile, about 1-4.5 mm wide, with the thinner hyphae often being contorted. Conidia are terminal and lateral, mostly sessile or on short, frequently conical protrusions or short side branches. Conidia are solitary but in close proximity to one another, 1-4 conidia developing on one hyphal cell, subhyaline, fairly thin- and smooth-walled, mostly subglobose, also clavate orobovoid, 1-celled, 2.5-11 x 1.5-6 mm, with broad basal scars (1-2 mm). Intercalary conidia are absent. Chlamydospores are absent.

5. Description of C1 strain. Colonies grow to about 55-60 mm

diameter in size on potato-dextrose agar in about 7 days; are white-cream-colored, felty, 2-3 mm high at the center; margins are defined, regular, fimbriate; reverse pale, cream-colored. Hyphae are hyaline, smooth- and thin-walled, little branched. Aerial hyphae are fertile, septate, 2-3 mm wide. Submerged hyphae are infertile. Conidia are terminal and lateral; sessile or on short side branches; absent; solitary, but in close proximity to one another, hyaline, thin- and smooth-walled, subglobose, clavate or obovoid, 1-celled, 4-10 mm. Chlamydospores are absent. Intercalary conidia are absent.

Conclusion. C1 is a strain of *Chrysosporium lucknowense* Garg 1966. For convenience the cellulase made by this strain is referred to herein as "C1" or "C1 cellulase."

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Example 4-- Assay for Cellulase Activity

The C1 strain was grown in 800 ml shake flasks rotated at 220 rpm and incubated at 28°C. The C1 strain was grown in saline Getchinson medium (See Table 2) (pH 7.5) containing 5 g/L of various nutrients, and in some cases with 2 g/L microcrystalline cellulose. One hundred ml of media were added to each flask.

Table 2. Getchinson medium for shake flasks

	g/L
KH ₂ PO ₄	1
KCl	0.1
MgSO ₄ ·7H ₂ O	0.3
NaCl	0.1
FeCl ₃	0.01
NaNO ₃	2.5

Combinations of glucose and microcrystalline cellulose, dextrose and microcrystalline cellulose, glycerol and microcrystalline cellulose, lactose and microcrystalline cellulose resulted in very low growth, formation of large aggregates of mycelium, and in the absence of cellulase activities (CMCase assay). The results are presented in Table 3. Additions of nitrogen organic sources, *i.e.*, peptone, corn steep liquor, or yeast extract enhanced growth and cellulase production and did not result in mycelium aggregates.

Lactose and yeast extract gave the highest cellulase production by C1. Similar results were obtained when the lactose and yeast extract were substituted with 25 g/L sweet beet pulp, 15 g/L barley malt, and 5 g/L wheat bran.

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Table 3. Effect of carbon and nitrogen sources on CMCase activity of C1 (shake flasks results)

Substrate	CMCase activity (units/ml) at pH 7.0		
	Days		
	3	5	7
Glucose+cellulose	0	0	0
Dextrose+cellulose	0	0	0
Glycerol+cellulose	0	0	0
Lactose+cellulose	0	0	0
Lactose+corn steep liquor	0	0	0.9
Lactose+peptone	10.7	7.4	14.8
Lactose+yeast extract	0	18.5	10.0
Cellulose+peptone	0.3	1.2	1.6
Cellulose+corn steep liquor	1.9	2.8	5.5

Example 5 -- Production of Cellulase for Stone Wash Tests

1. Production in shake flasks. C1 strain was grown in 800 ml shake flasks rotated at 220 rpm and incubated at 28°C for seven days. The growth medium 100 ml per flask was saline Getchinson medium (see Table 2) (pH 7.5) containing 25 g/L sweet beet pulp, 15 g/L barley malt, and 5 g/L wheat bran. The cell mass was separated by centrifugation and the cell-free supernatant was lyophilized and stored for further tests. C1 cellulase preparation #s 47.1.1 to 47.15.1 were produced in this manner. C1 preparation # 47.16.1 was produced by the same manner, but cell-free supernatant after centrifugation was ultrafiltrated using a 10 kDa cutoff membrane before lyophilization. C1 preparation #'s

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47.18.1 to 47.22.1 were produced by the same manner in shake flasks with Getchinson medium, but containing lactose (0.5 % w/v) and peptone (0.5 % w/v) instead of sweet beet pulp, barley malt and wheat bran. The cell mass was separated by centrifugation and the cell free supernatant was lyophilized and stored for further tests. Preparation #'s 47.1000, 47.1001, 47.2000 & 47.2001 were produced in shake flasks by the same manner as preparation #'s 47.1.1 - 47.15.1 except that they were produced using other *Chrysosporium* strains. Specifically, 47.2001 was produced by *Chrysosporium pannorum*, preparation 47.2000 was produced by *Chrysosporium pruinorum*, preparation 47.1001 was produced by *Chrysosporium keratinophilum* and preparation 47.1000 was produced by *Chrysosporium queenslandicum* (see Example 8). The protein content and activity fingerprints of these C1 preparations are shown in Table 4.

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Table 4. Protein content and activity fingerprints of C1 preparations and preparation #'s 47.1000, 47.1001, 47.2000 & 47.2001 which were prepared from other species of *Chrysosporium sp.*

Prepara- tion #	Protein, %	FPA, FPU/g	CMC- ase, U/g	Endo (visc), U/g	Avicel- lase, U/g	β -Glu- cosida- se, U/g
47.1.1	22	13	170	120	23	135
47.2.1	26	14	137	110	22	190
47.3.1	15	19	140	128	18	198
47.4.1	18	23	150	133	55	220
47.5.1	16	20	179	120	71	185
47.6.1	17	22	224	134	82	280
47.7.1	8	4	78	123	10	22
47.8.1	22	14	168	123	19	124
47.9.1	28	15	204	174	23	151
47.10.1	24	11	181	185	16	147
47.11.1	28	16	234	191	25	269
47.12.1	26	14	167	138	20	178
47.13.1	25	9	137	110	13	141
47.14.1	15	6	39	33	9	59
47.15.1	14	6	95	44	10	75
47.16.1	16	10	146	39	15	107
47.17.1	7	3	100	34	5	29
47.18.1	10	30	120	38	10	42
47.19.1	14	4	28	10	4	11
47.20.1	14	6	17	5	1	9
47.21.1	13	3	34	5	3	9
47.22.1	14	5	35	6	3	10
47.1000	18	4	31	35	6	89
47.1001	13	6	103	38	10	66
47.2000	10	3	78	31	7	67
47.2001	13	3	45	39	7	4
47.0325	50	155	4965	964	184	248
47.0528	67	111	13500	1782	232	423

2. Production in fermentors. C1 cellulase was produced in a 10-L

"ANKUM-1M" fermentor with Getchinson medium, lactose (0.5% w/v), peptone (0.5% w/v), and chloramphenicol (50 mg/mL). Initial volume of the nutrition

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medium was 7.0 L, final volume after fermentation was 7.3 L. The dissolved oxygen concentration (DO), agitation speed, aeration level, temperature, and pH were controlled. Fermentation was carried out as a batch-mode. The temperature of the fermentation was controlled at 28°C. The initial pH was 7.5 and was later maintained at that level by addition of NH₄OH (12% w/v). The aeration was at 4-5 L/minute and agitation at 400-500 rpm. The DO was maintained at above 50%. Samples (30 ml) were taken for analysis every 8 hours. At the end of fermentation, fungal biomass was separated by centrifugation (10,000g, room temperature, 20 minutes), and culture filtrate was lyophilized and stored for further tests. The results are shown in Table 5. Cellulase preparation # 47.17.1 was produced in this manner. Protein content and activity fingerprint of this C1 preparation is shown in Table 4.

Table 5. Production of C1 cellulase in 10-L fermentor

Time (h)	DO (%)	Reducing sugars (g/L)	CMCase (U/mL)
0	100	4.8	0
8	90	4.7	0
16	54	4.4	0
24	66	1.2	4
32	70	0.4	10
40	73	0.3	11.5
48	70	0.1	5
56	70	0	1

3. Production of C1 Preparation #'s 47.0325 and 47.0528. C1 cellulase preparation # 47.0325 was produced using the wild type C1 strain, preparation # 47.0528 was produced using an improved mutant obtained from the wild type C1 strain. These preparations were grown up fermentors under the conditions

described in Examples 13 and 15. Preparation 47.0325 was produced using a batch fermentation and 47.0528 was produced using a fed batch fermentation protocol.

5 4. Preparation of Humicola wild type preparation #'s 14.22.1 &
14.23.1 The wild type *Humicola grisea* var. *thermoidea* preparation # 14.22.1
was produced from the ATCC 16453 strain and the wild type *Humicola insolens*
preparation # 14.23.1 was produced from the ATCC 16454 strain. These
Humicola wild type preparations were produced in shake flasks using the same
10 method as described above for (Production in shake flasks) of C1 preparation #'s
47.1.1 - # 47.15.1.

Example 6 -- Comparison of C1 to Other Neutral Cellulases

15 The FPA, CMCase and endoglucanase activities of C1 enzyme
preparation # 47.0528 were compared to commercial *Humicola insolens* (Denimax
XT) and to wild ATCC-type Humicola (preparation #'s 14.22.1 *Humicola grisea*
var. *thermoidea* (ATCC 16453) & 14.23.1 *Humicola insolens* (ATCC 16454)
neutral cellulases. The results are given in the Table 6. The total activities of C-1
47.0528 are clearly higher than those of neutral cellulases from wild type
20 *Humicola* and from commercial *Humicola insolens* preparation. The specific
CMCase and endoglucanase activities (as units per gram of dry preparation or
units per gram of protein) of C-1 47.0528 are higher than those of all tested
Humicola preparations listed in Table 6. The specific FPA of C-1 # 47.0528 is
higher than the specific FPA of Humicola wild type preparations # 14.22.1 &
25 14.23 and slightly lower than the specific FPA of the *Humicola insolens*
commercial product Denimax XT. The pH and thermal stability of C1 cellulase
was similar to Denimax XT.

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Table 6. Comparison of *C1* and *Humicola* cellulases.

	Protein %	FPA	CMCase	Endo (visc)	FPA	CMCase	Endo (visc)
		unit/1 gram of dry preparation			units/1 gram of protein		
C1 (47.0528)	67	111	13,500	1782	165	20,115	2,655
Humicola sp. (# 14.23.1)	10	2	28	30	20	280	300
Humicola sp. (# 14.23.1)	10	1	11	19	10	110	190
Denimax XT (commercial)	13	25	450	99	192	3,460	761

(*) Activities were measured at pH 5.0 and 50°C

Example 7 --The effect of pH and temperature on activity and stability of C1 FPA and CMCase activities

The FPA and CMCase activities of C1 exhibit optimal stability and activity at about pH 6-7 and about 50-60°C; the pH optimum for CMCase activity is about 6.5, and the optimum temperature is about 55°C (see Tables 8,9). At pH 8.0 (50°C), CMCase possesses 80% activity, and FPA - 78% activity, at pH 9.0 (50°C), CMCase possesses 65% activity, and FPA - 52% activity (see Table 7.).

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**Table 7., the effect of pH on FPA and CMCase activities of C1 cellulase
(#47.19.1) at 50°C**

pH (50°C)	FPA (%)	CMCase (%)
4.0	50	60
4.5	68	70
5.0	75	78
5.5	80	80
6.0	92	90
6.5	100	100
7.0	95	95
7.5	90	92
8.0	78	80
8.5	60	75
9.0	52	65

The incubation time for the FPA assay was 60 minutes, the incubation time for CMCase assay was 5 minutes.

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Table 8. The effect of temperature on FPA and CMCase activities of C1 cellulase (#47.19.1), at pH 7.0

Temperature (C)	FPA(%)	CMCase (%)
40	45	50
45	60	55
50	70	65
55	100	100
60	70	60
65	40	30
70	20	25

The incubation time for the FPA assay was 60 minutes, the incubation time for the CMCase assay was 5 minutes.

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Table 9. Stability of CMCase of C1 cellulase (# 47.19.1) at 50 °C

Time (h)	CMCase activity remained (%)			
	pH 5.1	pH 7.2	pH 7.7	pH 8.5
0	100	100	100	100
0.5	100	98	95	85
1	100	95	93	55
2	100	82	78	32
3	100	78	65	25
5	100	75	45	15

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The CMCase of C1 exhibits high stability at optimal pH and temperature: For Example; at pH 7.2 and 50 C CMCase possesses 95 % activity after 1 hour and 75 % activity after 5 hours, at pH 7.7 and 50 C CMCase possesses 93 % activity after 1 hour and 45 % activity after 5 hours (See Table 9.).

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Example 8 -- Neutral and or alkaline Cellulase activity/ performance demonstrated in Other Strains of the Same Genera of *Chrysosporium*

Various strains of the *Chrysosporium* genus were tested for cellulase production. The full names and origins of these strains are described below.

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Strains obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, include:

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1. ATCC 44006 *Chrysosporium lucknowense*
2. ATCC 34151 *Chrysosporium pannorum*
3. ATCC 24782 *Chrysosporium pruinsum*

Strains obtained from the Russian Collection of Microorganisms (VKM) include:

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1. VKMF-2119 *Chrysosporium keratinophilum*
2. VKMF-2875 *Chrysosporium keratinophilum*
3. VKMF-2120 *Chrysosporium lobatum*
4. VKMF-2121 *Chrysosporium merdarium*
5. VKMF-2116 *Chrysosporium queenslandicum*
6. VKMF-2117 *Chrysosporium queenslandicum*
7. VKMF-2877 *Chrysosporium tropicum*

Two types of growth media were used in this study: medium A - Getchinson with sugar beet press, barley malt, and wheat bran; and medium B - Getchinson with peptone and lactose. The compositions of the media are described in Table 11.

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Table 11. Media for flasks studies

	<u>Medium A</u>	<u>g/L</u>	<u>Medium B</u>	<u>g/L</u>
	K ₂ HPO ₄	1	K ₂ HPO ₄	1
5	KCl	0.1	KCl	0.1
	MgSO ₄ ·7H ₂ O	0.3	MgSO ₄ ·7H ₂ O	0.3
	NaCl	0.1	NaCl	0.1
	FeCl ₃	0.01	FeCl ₃	0.01
	NaNO ₃	2.5	NaNO ₃	2.5
10	Sweet beet pulp	25	Lactose	5
	Barley malt	15	Peptone	5
	Wheat bran	5	pH	7.5
	pH	7.5		

15 The strains were grown in shake flasks at 220 rpm and at 28°C. Samples of each strain grown in Medium A were taken for analysis after 6 and 7 days of culture. Samples of strains grown in Medium B were taken after 5 days in culture. All samples were assayed for CMCase activity at pH 5 and 7. The results of the CMCase assay are shown in Table 12.

Table 12. Cellulase production by different strains of *Chrysosporium*

Strains	medium A (6 days)			medium A (7 days)			medium B (5 days)		
	RS		CMCase	RS		CMCase	RS		CMCase
	pH 5	pH 7		pH 5	pH 7		pH 5	pH 7	
1 VKMF 2117	2.7	0	0.46	2.6	0.00	0.00	2.3	0.21	0.09
2. VKMF 2116	1.1	0.22	0.04	0.2	0.38	0.61	4.0	0.58	0.59
3. VKMF 2121	1.9	0	0.57	1.1	0.25	0.10	2.5	0.25	0.09
4. ATCC 24782	3.4	0.33	1.40	1.9	1.85	0.11	3.0	1.10	0.06
5. ATCC 34151	1.0	1.54	0.90	0.9	0.17	0.20	4.3	0.81	0.90
6. ATCC 44006	4.4	0.21	0.49	2.0	0.68	0.34	2.5	1.29	0.06
7. VKMF 2119	4.1	0	0.08	2.7	0.29	0.00	3.8	0.95	0.04
8. VKMF 2120	4.5	0	0.17	2.3	0.23	0.00	2.3	0.12	0.00
9. VKMF 2875	1.6	0	1.01	1.7	0.00	0.00	3.8	1.96	0.05
10. VKMF 2877	2.4	0	0.03	0.8	0.22	0.00	5.0	0.43	0.00
11. C1 (VKMF 3500D)	2.9	1.70	1.65	nt	nt	nt	0.1	0.89	0.80

RS = concentration of reducing sugars in the fermentation medium at the end of fermentation, g/L (Nelson-Somogyi method).

pH 5, pH 7 = the values of pH under which the CMCase activity of the fermentation broth was assayed.

CMCase activity in U/ml.

nt = not tested

In the cases of strains ATCC 34151 *Chrysosporium pannorum*, ATCC 24782 *Chrysosporium pruinosum*, VKMF-2875 *Chrysosporium keratinophilum*, VKMF 2116 *Chrysosporium queenslandicum* the cell mass was separated by centrifugation and cell free supernatant concentrated from 5 liters to 0.5 liter by ultrafiltration using 10 kDa cut-off membrane. Then the ultrafiltrated concentrate was lyophilized and stored for tests.

The following #-s of cellulase dry preparations were used:

47.2001 - ATCC 34151 *Chrysosporium pannorum*,

47.2000 - ATCC 24782 *Chrysosporium pruinosum*,

47.1001 - VKMF-2875 *Chrysosporium keratinophilum*.

47.1000 - VKMF 2116 *Chrysosporium queenslaandicum*.

Protein content and activity fingerprints of these preparations are given in Table 4.

5 Example 9 -- Stone Wash Tests

A. Tests with 2-L special washing machine. This system assesses the stone wash performance characteristics related to abrasion and backstaining using only small amounts of enzyme.

Desizing. Forty pieces (30 g each, 25 x 20 cm) of denim fabric
10 (roll) (1.2 kg) were desized in a household washer at 60°C for 20 minutes using a fabric:liquor ratio of 1:6 (7.2 L) and 0.5 g/L (3.6 g) Sandoclean PC liquid (nonionic washing and wetting agent on base of ethoxylated fatty alcohols with an average of 6 moles of ethylene oxide, 1 g/L (7.2 g) Sirrix 2UD (acidic buffered sequestration) and 1 g/L (7.2 g) Bactosol TK liquid (high temperature stable alpha-
15 amylase) at a pH of about 5 to 6. After 20 minutes, the liquor was drained and the pieces washed for 5 minutes with cold water (14 L) liquid ratio 1:10. The pieces were dried at 40°C and used as a stock of comparable samples for the determination of cellulase activity

 The cellulase treatment of the garment pieces was carried out in a
20 washing machine consisting of an inner drum of 29 cm diameter drum - 10.6 l total volume (drum rotates at 20 rpm - five turns left - five turns right). Each piece of fabric was sewn together with 4 rubber stoppers prior to the cellulase treatment to give a garment package that ensured that the mechanical effect occurred mainly on the darker outer side of the garment. Each drum was filled
25 with one package and 10 additional rubber stoppers.

 The general wash conditions were: 30 g desized denim jean fabric, cellulase in 0.02 M citrate buffer, 50°C, 60 minutes, garment:liquor ratio 1:4. After the cellulase treatment the package was washed with hot water (50 °C) (garment:liquid ratio 1:20) for 5 minutes and dried for evaluation.

30 Application trials were conducted using various C1 cellulase preparations

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along with other cellulase preparations prepared from different species of *Chrysosporium* as well as the commercial Novo Nordisk neutral cellulase products, Denimax XT (US Patent # 4,435,307) and Ultra MG (WPO 91/17243). These application trials were set up to evaluate the stone wash performance characteristics of C-1 as well as several other species of *Chrysosporium* cellulases vs Novo's commercial neutral cellulases. The trials were run at neutral and alkaline pH's (6.5, 6.7, 7.0, and 8.0). The results are presented in Table 13. Garments treated with various C1 and other *Chrysosporium* cellulase preparations showed similar wash performance characteristics to those of the commercial neutral cellulases Denimax XT and Denimax Ultra MG. The C-1 and other *Chrysosporium* cellulase preparations showed good softening effect, bleaching/overall shade reduction, abrasion levels as well as low backstaining values when run under neutral and alkaline pH conditions. Datacolor measurement is based on the degree of lightness of the sample (reflectance). The sample is exposed to white light (2 pulsed Xenon flash lamps) and the remission is detected between 400 and 700 nm with 16 diodes. Reflectance from the front side, the higher value the more abrasion. Reflectance from the back side, the higher value the more backstaining.

Table 13., Enzyme Wash With Special 2 Liter Machine (135 grams of denim per run)

Enzyme	Amount g	%OWG	CMCase		Endo (visc)		°C	Liquo r ratio	pH	Time (min)	Buffle r	Dataco lor Abrasi on	Dataco lor Backst ng
			U/g	/ru n	U/g	/ru n							
C-1 47.11.1	1.995	1.5	234	474	191	381	50	1:11	7. 2	60	0.02M P	13.1	1.8
Denimax XT	0.133	0.10	450	60	99	13	50	1:11	7. 0	60	0.02M P	13.1	2.4
C-1 47.12.1	2.100	1.5	167	338	138	290	50	1:11	6. 7	60	0.02M P	14.2	1.8
Denimax XT	0.420	0.30	450	182	99	42	50	1:11	6. 6	60	0.02M P	14.0	2.3
C-1 47.9.1	3.29	2.44	204	671	174	572	50	1:11	6. 7	60	0.02M P	17.1	1.8
C-1 47.16.1U	2.3	1.7	146	336	39	90	50	1:11	6. 7	60	0.02M P	16.2	2.3
47/1000.1	7.0	5.19	48	336	14	98	50	1:11	6. 5	60	0.02M P	12.9	2.1

Enzyme	Amount g	%OWG	CMCase		Endo (visc)		°C	Liquo r ratio	pH	Time (min)	Buffle r	Dataco lor Abrasi on	Dataco lor Backst ng
			U/g	/ru n	U/g	/ru n							
47/2001.1	7.15	5.30	47	336	20	143	50	1:11	6.5	60	0.02M P	14.7	1.7
Denimax UltraMG	0.132	0.10	134	18	243	32	50	1:11	7.3	60	0.02M P	14.1	3.5
C-1 47.19.1	7.14	5.29	28	200	9	64	50	1:11	6.5	60	0.02M P	14.7	1.9
C-1 47.0325	0.068	0.05	4965	338	964	66	50	1:11	7.0	60	0.02M P	15.1	2.3
Denimax XT	1.0	0.74	450	450	99	99	50	1:11	6.5	60	0.02M P	19.1	2.8
C-1 47.0528	0.08	0.05	4800	384	178	143	50	1:11	6.5	60	0.02M P	18.5	3.0
C-1 47.0325	0.136	0.10	4965	675	964	131	50	1:11	6.0	60	0.02M P	18.3	3.8
C-1 47.0325	0.136	0.10	4965	675	964	131	50	1:11	7.0	60	0.02M P	18.9	3.2

Enzyme	Amount g	%OWG	CMCase		Endo (visc)		°C	Liquo r ratio	pH	Time (min)	Buffle r	Datacolor Abrasion	Datacolor Backst ng
			U/g	/ru n	U/g	/ru n							
C-1 47.0325	0.136	0.10	4965	675	964	131	50	1:11	8.0	60	0.02M P	16.7	2.5
Humicola 14.22.1	9.18	6.80	28	257	30	275	50	1:11	6.7	60	0.02M P	14.9	1.3
Humicola 14.23.1	9.18	6.80	11	101	19	174	50	1:11	6.7	60	0.02M P	12.5	1.5
T. reesei CP	0.30	0.22	9190	273	200	600	50	1:11	4.8	60	0.02C A	17.5	8.0
Blank	0.00	0.00	0	0	0	0	50	1:11	6.5	60	0.02M P	9.1	n/a

0.02MP = Phosphate Buffer System

0.01CA = Citric Acid Buffer System

T. reesei CP = Commercial acid cellulase product produced from Trichoderma reesei.
Datacolor Abrasion = reflectance from the front side, the higher the values, the more abrasion, blank = 9.1

Datacolor Backstng = reflectance from the back side, the lower the values, the lower the back staining

% OWG = for example for 1% OWG, 1 lb of enzyme is used on 100 lbs of garment

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B. Tests with 35 lb washing machine. Application Trials were run in a 35 lb washing machine (35 lb washing machine brand is Milnor - washer RPM is 30). Load size is 2400 g (3 garments), garments used are Levi's 505 jeans. Water level for cellulase bath is 15 L for a liquor ratio of 6.25:1 (low). The water level for all other baths is 24 L for a liquor ratio of 10:1 (Med). The buffering system used is MAP - monoammonium phosphate and DAP - diammonium phosphate to maintain the pH of 6.7 during the cellulase bath. In Trials 4, 5, 6 & 7 a nonionic detergent was added to the cellulase bath, it is known that adding a detergent to the cellulase bath will help in reducing the backstaining on the garments. Zeke is a desizing product. SSCE is Superscour, a nonionic detergent (Zeke and Super Scour are commercial specialty textile chemical products offered by CPN International, Ltd., Inc of Jupiter, Florida). One Example of the Wash Formulas used in these trials is Trial 2. below;

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Wash Formula - Trial 2.

(C-1 47.0528)

	Load (g)	2400 (3 gmts)	Fabric:	Denim	Formula Time:	1:30		
	Machine:	35# Milnor	Weight:	14.5 oz	Developed by:			
Step	Operation	Time (min)	Level	Temp (F)	Chemical	Amount	%OWG	pH
1	Desize	10	Med	150	Zeke	48 g	2	
2	Drain Balance							
3	Rinse	2	Med	140				
4	Drain Balance							
5	Rinse	2	Med	130				
6	Drain Balance							
7	Abrasion	75	Low (15L)	125	MAP	29 g	buffer	6.7
					DAP	10 g		
					C-1	1.2 g	0.05	
8	Drain Balance							
9	Wash	10	Med	160	SSCE	24 g	1	
10	Drain Balance							
11	Rinse	3	Med	120				
12	Drain Balance							
13	Rinse	3	Med	100				
14	Drain Balance							
15	Rinse	3	Med	100				
16	Drain Balance							
17	Extract	2						

In the example above, and in commercial use, one skilled in the art will appreciate that the use of pumice stones in the stonewash process will enhance the

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overall stonewash effect on the garments.

The results in Table 14. show that the C1 cellulase preparations # 47.0325 and # 47.0528 performed better in terms of the overall level of abrasion achieved on the garments and well within the range of the backstaining level of the other commercial neutral cellulase products tested.

Table 14., Comparison of C-1 Cellulase Preparations 47.0325 & 47.0528 To Commercial Neutral Cellulases Denimax XT & BTU 202 - 318 (which contains Denimax XT)

Trial	Cellulase	% OWG	Wt(g)	Detergent	T(°F)	pH	t(min)
1	Denimax XT	0.50	12.0	no	130	6.7	75
2	C-1 47.0528	0.05	1.2	no	125	6.7	75
3	C-1 47.0325	0.10	2.4	no	125	6.7	75
4	Denimax XT	0.50	12.0	yes	130	6.7	75
5	C-1 47.0528	0.05	1.2	yes	125	6.7	75
6	C-1 47.0325	0.10	2.4	yes	125	6.7	75
7	BTU 202-318	2.50	60.0	yes	130	SB	75

ABRASION

(Most to Least)

Trial 5

Trial 6

Trial 2

Trial 3

Trial 4

Trial 1

Trial 7

BACKSTAINING

(Least to Most)

Trial 4

Trial 5

Trial 6

Trial 3

Trial 1

Trial 2

Trial 7

Figure Legend for Table 14 All of the trials in table 14. were cleaned up with

Super Scour (nonionic detergent) at 1.0 % OWG, 160 F for 5 minutes. SB = Self

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Buffered - the commercial product "ROCKSOFT" BTU 202-318 contains Denimax XT, detergent and a buffer system as well as other additives to help enhance the stone wash performance of this commercial product.

5 C. Tests with 60-L special washing machine. Whole denim garments were desized as described for the 2-L washing machine tests. Each wash test was made with 1 pair of jeans (700 g), 2.8 L liquid (fabric:liquid ratio 1:4). All jeans were from the same dye lot. They were prewashed using an oxidation method for 15 minutes, then dried. Blue jeans washed at neutral pH with formulated C1 cellulase preparations 47.0325 using 2.4 grams per trial and 47.0528 using 1.5 grams for 10 one trial and using 1.0 gram for a second trial were compared directly against blue jeans washed under neutral pH conditions and similar formulations using Denimax XT at 12 grams per trial and two other commercial neutral cellulases; Bactosol JE using 2.0 % OWG and BTU 202-318 using 2.0 % OWG (Bactosol JE and BTU 15 202-318 contain Denimax XT, buffer, detergent as well as other additives to enhance their wash performance). Table 15. shows that the blue jeans from all three C-1 trials outperformed the three commercial neutral cellulase products in terms of the level of abrasion achieved as well as the overall color reduction of the garments. The level of backstaining on the blue jeans from all six trials was very 20 good, they were very similar to one another and what one would expect and see when using Novo's neutral cellulase Denimax XT. The backstaining values for all three of these C-1 trials were within the range of the backstaining values as shown in Table 13. The finished garments from these trials and the trials as rated and shown in Table 14 above were rated in a blind study by four independent groups, 25 of three or more people per group. The people that made up each of these groups are considered to be skilled in the art of stonewashing. They were asked to place each of the garments in the following order: (1) Greatest overall abrasion and color reduction to least overall abrasion and color reduction; and (2) Backstaining, lowest level of backstaining to highest level of backstaining (See Table 15).

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Table 15

TRIAL	ENZYME	DOSAGE	BUFFER	DETERGENT	ABRASION/ COLOR REDUCTION	BACKSTAINING
Trial A	C-1 47.0528	1.5 grams	Phosphate	Yes	++++++	5
Trial B	C-1 47.0528	1.0 grams	Phosphate	Yes	+++++	2
Trial C	C-1 47.0325	2.4 grams	Phosphate	Yes	+++++	4
Trial D	Denimax XT	12.0 grams	Phosphate	Yes	++++	3
Trial E	BTU 202-318	2.0% OWG	Phosphate	Yes	+++	6
Trial F	Bactosol JE	2.0% OWG	Citrate	Yes	+++	1

Legend for Table 15:

Abrasion/Color Reduction - ++++++ (+6) best (= > ++++ (4) is considered good and was comparable to commercial natural cellulases (e.g. - Denimax XT)

Backstaining - The lower number the better (all jeans were judged to be within the range of backstaining as found when using Novo's Denimax TX). Neutral cellulase significantly decreased backstaining compared with traditional acid cellulases such as *Trichoderma* (see Example 13)

%OWG - %Of Weight of Garment, for example for 100 lbs of jeans dryweight at 1% OWG, 1 lb of enzyme is used.

D. Light reflectance. Another test to evaluate backstaining is to measure the light reflectance of a treated fabric. At the end of washing treatment, jeans samples were analyzed using a reflectometer at two different wavelengths: (1) the higher the signal detected at 680 nm (measured at the outside of the jeans), the lower the backstaining; and (2) the higher the signal detected at 420 nm (measured at the inside of the jeans), the lower the backstaining. Table 16. compares the reflectance values of denim jeans after treatment with commercial cellulases from Novo Nordisk and Genencor International to C-1 preparation # 47.6.1.

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Table 16.

Enzyme	680 nm	420 nm
Denimax L (neutral cellulase, Novo)	23	20
5 Primafast 100 (acid cellulase, Genencor)	20	13
C1 47.6.1 (neutral/alkaline cellulase)	22	18

The light reflectance values for the C1 cellulase were similar to those obtained with Novo Nordisk's commercial product Denimax L, a neutral cellulase, at both 680 and 420 nm and the light reflectance values for C1 cellulase were significantly better than those obtained with Genecor's commercial product Primafast 100, a acid cellulase, at both 680 and 420 nm.

E. Tests in semi-industrial washing machine.

Test #1.

2 Jeans, weight 1343 gr

Water ratio 6:1

pH 5.5

Temp. 54°C

Enzyme: C1 (preparation # 47.6.1) 12 gr
(0.9%)

Abrasion time 90 minutes

Drop bath

Rinse 5 minutes with 1% non-ionic detergent at
66°C

Drop bath

Rinse cold

Drop bath

Soften for 5 minutes with cationic softener at
49°C

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Extract and dry.

Test #2. The same procedure as Test #1, above, except Denimax 700 T (2% OWG 28.9 gr) enzyme was used and wash conditions were conducted at pH 7.0, 54°C.

C1 cellulase was compared to Denimax 700 T, a neutral cellulase commercial product made by Novo. All jeans were from the same dye lot. They were prewashed for 15 minutes using an oxidation method then dried.

The jeans treated with C1 cellulase preparation # 47.6.1 showed slightly less abrasion and lower backstaining than the jeans treated with Denimax 700T cellulase.

Example 10---C1 Cellulase as an Additive to Laundry Detergent

A. Soil Release from Cotton

Wash performance of C1 cellulase preparation # 47.9.1 was tested using the wash-performance procedure PW 9406 (Solvay). Soil (ink) release from cotton fabric was tested by Delta Reflectance (%). Wash test compared a C1 cellulase preparation (# 47.9.1) to Celluzyme 0.7 T from Novo Nordisk in the presence and absence of alkaline protease Opticlean L500. The results of this test are shown in the Table 17.

C1 cellulase has soil release properties from ink soiled cotton at neutral pH in a color type detergent as the cellulase enzyme from *Humicola insolens*.

Table 17. Detergent wash test with C1 cellulase(*)

Enzyme tested (pH 7.0)	CMCase dosage (U/l)	Reflectance Data (%)	
		1	2
Celluzyme 0.7 T	200	3.68	4.75
Celluzyme 0.7 T	500	2.68	4.07
5 Celluzyme 0.7 T + 5000 DU/l (**)	200	2.07	3.13
Celluzyme 0.7 T + 5000 DU/l (**)	500	2.08	3.22
C1 # 47.9.1	200	2.18	2.88
C1 # 47.9.1.	500	2.77	3.72
C1 #47.9.1 + 5000 DU/l (**)	200	1.15	1.91
10 C1 #47.9.1 + 5000 DU/l (**)	500	2.81	3.30
None (control)	none	0	0

AADU = Du = Delft unit, Du/l = Delft unit per liter

(*) 40°C, 45 min, drying at 68°C, 75 min

(**) Alkaline protease Opticlean L500

B. The Stability of C1 Cellulase with Serine Proteases

As serine proteases, a trypsin (3.2 μ M, from Bovine Pancreas, activity 10,000-13,000 N-benzyl-L-arginine ethylester (BAEE)/mg, Sigma T-8253) and an α -chymotrypsin (8 μ M, from Bovine Pancreas, 40-60 U/mg, Sigma C-4129) were used.

The proteases were incubated with C1 cellulase at 20°C and pH 7.0. Chymotrypsin did not decrease C1 activity for 12 hours and trypsin led to a slight decrease (around 20%) of C1 activity, see Table 18.

Trypsin and chymotrypsin did not significantly change the stability of C1 CMCase at pH-s 4.5 and 7.0 at 50° and 57°C, see Table 18.

Table 18. The effect of proteases on CMCase activity of C1 cellulase (# 47.9.1)

Protease	Temperature (°C)	pH	Incubation time (h)	CMCase activity remaining (%)
None (control)	20	7.0	12	70
+ Chymotrypsin	20	7.0	12	70
+ Trypsin	20	7.0	12	50
None (control)	50	4.5	3	100
+ Chymotrypsin	50	4.5	3	100
+ Trypsin	50	4.5	3	100
None (control)	50	7.0	3	78
+ Chymotrypsin	50	7.0	3	60
+ Trypsin	50	7.0	3	68
None (control)	57	4.5	3	62
+ Chymotrypsin	57	4.5	3	60
+ Trypsin	57	4.5	3	62
None (control)	57	7.0	3	30
+ Chymotrypsin	57	7.0	3	30
+ Trypsin	57	7.0	3	30

C. The Effect of Citrate, EDTA, Tween-80 and Persulfate on CMCase activity

Changing from acetate to citrate buffer (a chelating agent) did not effect the of C1 CMCase activity (molarity of buffers - 0.1 M, pH 4.5, 50 and 57°C), see Table 19.

EDTA (Ethylene Diamine Tetraacetic Acid) (5 mM) as a chelating agent at pH 4.5 and 50°C did not change CMCase activity. At pH 4.5 (57°C) and at pH 7.0 (50°C) EDTA caused slight decreases in CMCase activity. At pH 7.0 and 57°C, EDTA caused slight increase in CMCase activity, see Table 19.

Non-ionic detergent Tween-80 (3 g/L, polyoxyethylene sorbitane

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monooleate), did not change CMCase activity of C1 (at pH-s 4.5 and 7.0 and at 50 and 57°C, see Table 19.

Oxidizing agent persulfate (3 g/L) did not change CMCase activity of C1 (at pH-s 4.5 and 7.0 and at 50 and 57°C), see Table 19.

5

C1 CMCase is resistant to serine proteases (trypsin and chymotrypsin), chelating agents (EDTA, citrate), non-ionic detergent (Tween-80) and to oxidizing agent (persulfate).

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Table 19. The effect of citrate, EDTA, Tween-80 and persulfate on activity of C1 cellulase (# 47.9.1). Incubation time - 3 hours.

Effector	Concentration	Temperature (°C)	pH	CMCase activity remaining (%)
None (control)	---	50	4.5	100
Citrate	0.1 M	50	4.5	100
EDTA	5 mM	50	4.5	100
Tween-80	3 g/L	50	4.5	100
Persulfate	3 g/L	50	4.5	97
None (control)	---	57	4.5	62
Citrate	0.1 M	57	4.5	65
EDTA	5 mM	57	4.5	60
Tween-80	3 g/L	57	4.5	68
Persulfate	3 g/L	57	4.5	65
None (control)	---	50	7.0	78
EDTA	5 mM	50	7.0	50
Tween -80	3 g/L	50	7.0	52
Persulfate	3 g/L	50	7.0	50
None (control)	---	57	7.0	30
EDTA	5 mM	57	7.0	38
Tween-80	3 g/L	57	7.0	25
Persulfate	3 g/L	57	7.0	30

Example 11 -- Stone wash tests of cellulase samples produced by different strains of *Chrysosporium*

Preparations #-s 47.1000, 47.1001, 47.2000 and 47.2001 produced by different strains of *Chrysosporium* were used for wash test with 2-L special wash machine at pH 6.5, 50°C, during 60 min with 135 g of desized denim jean fabric.

Total amount of CMCase activity per trial was constant and equal to 336 U/run.

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After drying abrasion and backstaining of garment was evaluated by Datacolor measurement. The results are presented in Table 20. The results show that cellulases produced from different strains of *Chrysosporium* demonstrate similar wash performance at neutral pH in terms of abrasion and backstaining levels to the cellulases produced by the C-1 species *Chrysosporium lucknowense* Garg 1966.

Table 20. Stone wash activity of cellulase preparations from different strains of *Chrysosporium*

Preparation #	Abrasion (*)	Backstaining (**)
47.1000	12.3	1.6
47.1001	12.1	1.6
47.2000	14.2	1.7
47.2001	14.6	1.6

(*) - reflectance from front side, the higher value the more abrasion, blank = 9.1
 (**) - reflectance from back side, the higher value the more backstaining

Example 12. -- Purification of Cellulase Components

1. Selection of the C1 samples for purification. The C1 cellulase preparation #47.11.1 was chosen for further purification in view of the fact that 47.11.1 possessed (i) high protein content; (ii) high FPA and CMCase activity (see Table 4).

2. Isolation and purification of C1 complex component. The first purification step included ion exchange chromatography on a DEAE-Toyopearl column (TosoHaas, Japan). Dry C1 cellulose preparation (1.5 g) was dissolved in 15 mL of 0.01 M Na-phosphate buffer, pH 7. The solution was centrifuged and the supernatant desalted using an Acrylex P-2 column. The desalted sample was then applied to the DEAE-Toyopearl column (1.5 x 30 cm) in 0.03 M phosphate buffer,

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pH 4.7 and adsorbed proteins were eluted in 0 - 0.2 M NaCl gradient with flow rate of 1 mL/min. Three pooled fractions were obtained -- the non-bound (NB) fraction was eluted in the start buffer, Fractions I and II were eluted across a 0 - 0.2 M NaCl gradient. All fractions possessed cellulolytic activities.

5 3. SDS-PAGE of protein fractions. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the NB Fraction included proteins with molecular weights from 30 and 70 kD. Fraction I includes proteins with molecular weights from 25 to 100 kD and Fraction II contained proteins with molecular weights from 35 and 100 kD. SDS-PAGE was carried out with a 10% separating gel (100 X 80 X 0.75 mm) under denaturing conditions. Reagents and kits were obtained from Bio-Rad (USA). Coomassie brilliant blue R-250 in 25% trichloroacetic acid was used for protein staining.

10 4. IEF of Protein Fractions. After isoelectric focusing (IEF), the NB Fraction includes proteins with isoelectric points (pI's) of from 4.6 to 8.0. Fraction I contains proteins with pI values from 3.2 to 5.5, and Fraction II contains proteins with pI from 3.0 to 5.5. Isoelectric focusing was carried out with 7% PAAG in mini-IEF Model 111 (from Bio-Rad). Reagents and kits were obtained from Bio-Rad (USA). Coomassie brilliant blue R-250 in 25% trichloroacetic acid was used for protein staining.

20 5. pH-dependencies of CMCase activity of protein fractions. Table 21 represents the pH dependencies of CMCase and RBB-CMCase activities of Fractions NB, I and II of C1 cellulase. The following buffer systems were used: acetate buffer (pH 4-5), phosphate buffer (pH 6-8), and carbonate buffer (pH 8.5-10). In addition, a universal buffer system was used which consisted of acetate, borate, and phosphate (pH 4-10).

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Table 21. The effect of pH on CMCase and RBB-CMCase activities of C1 protein fractions

pH	CMCase activity, 50°C (%)		
	NB Fraction	Fraction I	Fraction II
4.0	85	70	95
4.5	95	85	100
5.0	100	90	95
5.5	90	100	90
6.0	80	90	80
6.5	70	85	80
7.0	65	85	80
7.5	60	65	75
8.0	50	60	60
8.5	45	50	50
9.0	30	45	40
9.5	10	40	32

pH	RBB-CMCase activity, 50°C (%)		
	NB Fraction	Fraction I	Fraction II
4.5	65	95	100
5.0	100	100	95
5.5	95	100	90
6.0	95	95	90
6.5	80	87	90
7.0	80	85	87
7.5	60	65	85

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8.0	50	60	70
8.5	45	60	60
9.0	30	50	40
9.5	10	40	32

CMCase and RBB-CMCase activities of the NB, I and II Fractions following DEAE-Toyopearl ion exchange chromatography had a non-symmetric bell-type pH profile. CMCase activity of the NB Fraction showed a maximum at pH 4.5 - 5.5 and 50% of maximal activity at pH 8.0. RBB-CMCase activity of the NB Fraction had a maximum at pH 5.0 - 6.0 and 50% of maximal activity at pH 8.0. CMCase activity of Fraction I had a maximum at pH 5.0 - 6.0 and 50% of maximal activity at pH 8.5. RBB-CMCase activity of Fraction I had a maximum at pH 4.5 - 7.0 and 50% of maximal activity at pH 8.5. CMCase activity of Fraction II had a maximum at pH 4.0 - 5.5 and 50% of maximal activity at pH 8.5. RBB-CMCase activity of Fraction II had a maximum at pH 4.5 - 7.5 and 50% of maximal activity at about pH 8.5.

6. Stability of CMCase of protein Fraction I (after DEAE-Toyopearl).

Table 22 shows temporal CMCase activity curves of Fraction I after DEAE-Toyopearl ion exchange chromatography at different pH (5.2-8.7) and 50°C. CMCase activity of Fraction I was most stable at pH 5.2-7.2 (between about 30% and 45% of activity was lost over 3 hours). At pH 7.7, 60% of activity was lost after about 1 hour, whereas at pH 8.3 and 8.7, 50% of activity was lost after about 0.5 hour. At pH 8.3, 100% of CMCase activity was lost after 3 hours, and at pH 8.7, 100% of activity was lost after 2 hours.

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Table 22. Stability of CMCase of a protein fraction I after DEAE-Toyopearl at 50°C

Time (h)	CMCase activity remained (%)				
	pH 5.2	pH 7.2	pH 7.7	pH8.3	pH 8.7
0	100	100	100	100	100
0.5	97	75	65	50	50
1	90	55	40	25	15
2	40	50	35	10	0
3	30	45	25	0	0

7. Properties of Fractions after Ion Exchange Chromatography on DEAE-

Toyopearl. Adsorption experiments using Avicel (micro crystalline cellulose) as a substrate demonstrated that fractions I and II did not bind to a crystalline substrate and the NB Fraction bound to Avicel with a distribution coefficient of 0.2 L/g. Specific activities of the NB, I and II Fractions toward different substrates are presented in Table 23. All three fractions possessed CMCase, endoglucanase, avicelase, β -glucanase and xylanase activities, but the NB Fraction had no β -glucosidase activity, contrary to Fractions I and II. The Micro Denim Wash test for Fractions NB, I and II showed that Fractions I and II possessed approximately equal activity on denim at pH 7 while the NB Fraction showed lower activity (according to the Micro Denim Wash test).

Table 23. Specific Activities of Fractions After Ion Exchange
Chromatography on DEAE-Toyopearl

Specific Activities, U/mg Protein								
Fractions	CMCase	Endoglu -canase	Avicelase	β -gluco -sidase	β -gluc -anase	Xylan -ase	Micro Denim Wash Test	
NB	9.9	6.2	0.18	0.0	11.0	5.3	+	
I	3.7	2.3	0.02	3.6	1.9	0.7	++	
II	0.8	0.2	0.02	0.02	1.2	0.04	++	

8. Micro Denim Wash Test. This test was carried out using 20 mL of buffered enzyme solution having a preset level of CMCase activity. Real Indigo-denim stained swatches were treated at 50°C for 60 minutes in the conditions of excessive mechanical stress (abrasion). The level of cellulase performance was evaluated by panel score according to the color reduction after the samples were dried. Color measuring instruments and software could also be used. More "+" values indicate better abrasion.

9. Further Purification of Proteins from Fraction I After Ion Exchange Chromatography on DEAE-Toyopearl. Desalted Fraction I (25 mL, 0.8 g/l of protein) was subjected to Macro Prep Q ion exchange chromatography. The MacroPrep Q column (1.5x10 cm) was equilibrated with 0.03 M acetate buffer, pH 4.7, and the adsorbed proteins eluted in 0 - 0.1 M NaCl concentration gradient. Three fractions, I.1, I.2, and I.3, were collected. Fraction I.2 showed the highest Micro Denim Washing activity at pH 7 and fraction I.3 showed the lowest. SDS-PAGE results showed that Fractions I.1 and I.2 differed by the presence of a low molecular weight protein (25 kD) in Fraction I.2, which might have accounted for the stonewashing activity. Fraction I.1 had a pI of 4.2 as shown by IEF

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measurements. Fractions I.1 and I.3 had too low a protein content to permit further study. Therefore, Fraction I.2 was used in further purification.

The next purification step involved chromatofocusing on a Mono P column. Fraction I.2 was equilibrated with 0.03 M imidazole buffer, pH 6.8, and applied to the column. Adsorbed proteins were eluted with Polybuffer 74 (1:8), pH 4.0, whereupon 2 major peaks in protein and activity profiles were observed. The first peak, designated Peak A, showed lower specific CMCase activity (2.5 Units/mg) compared to Peak B (3.6 Units/mg). Native polyacrylamide gel electrophoresis showed the presence of 2 protein bands in Peak A, with the higher molecular weight protein band being active toward CMC stained with Congo Red. One active protein band under native conditions was observed in Peak B. SDS-PAGE data showed that Peak A included 2 major proteins (60 kD and 70 kD) and Peak B contained one major protein (25 kD) and one minor protein (27 kD). The fraction collected within Peak B was designated as 25 kD-endoC1 and was used in further studies. Table 24 shows the specific activities of 25 kD-endoC1 toward different substrates. The 25 kD-endoC1 had CMCase, RBB-CMCase, endo-glucanase, FPA, avicelase, β -glucanase, and xylanase activities but did not show β -glucosidase activity. This combination of different activities shows that 25 kD-endoC1 is an endoglucanase. The pH optimum of CMCase and RBB-CMCase activities is approximately 6.0 (Table 24). The 25 kD-endoC1 possessed high stonewashing activity (according to a Micro Denim Wash test using cotton swatches) (see Table 24).

During elution of Peak A from the Mono P column a number of fractions were recovered that differed in the ratio of 60 kD to 70 kD proteins, especially a fraction designated 70(60) kD-C1 that included predominantly a 60 kD protein and a fraction designated 70 kD-endoC1 with predominantly a 70 kD protein. Specific activities of these fractions toward different substrates are presented in Table 24. As is seen therein, the 70(60) kD-C1 fraction possessed low specific endoglucanase (0.5 U/mg) and a high specific avicelase (0.31 U/mg) activities compared to the 70 kD-endoC1 fraction (2.8 U/mg endoglucanase and 0.18 U/mg avicelase) and had

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very low β -glucosidase activity. Specific activities toward FP, β -glucan, and xylan of the 70(60) kD-C1 fraction were low (see Table 24) and only cellobiose was formed as a product of avicel hydrolysis. Stonewashing activity (according to a Micro Denim Wash test using cotton swatches) of the 70(60) kD-C1 fraction was low (see Table 24). These data show that the 60 kD protein from Fraction I after ion exchange chromatography on DEAE-Toyopearl can be designated as a cellobiohydrolase. The pH optimum of 70(60) kD-C1 (toward CMC and RBB-CMC) was approximately 5.0 (Table 24).

The 70kD-endoC1 had high specific CMCase, RBB-CMCase, endoglucanase, FPA, β -glucanase and xylanase activities and possessed some avicelase and β -glucosidase activity (Table 24). The 70kD-endoC1 also possessed relatively high stonewashing activity (according to the Micro Denim Wash test using cotton swatches) (see Table 24). The 70kD-endoC1 from fraction I after ion exchange chromatography on DEAE-Toyopearl appears to be an endoglucanase. As seen from Table 24, the pH optimum for 70 kD-endoC1 is approximately 6.0 for both the CMCase and RBB-CMCase activities.

10. Further Purification of Proteins from Fraction II after Ion Exchange Chromatography on DEAE-Toyopearl. Fraction II, obtained as a result of DEAE chromatography, was divided into 3 fractions (Fraction II.1, II.2, and II.3, respectively) using a longer 0-0.2 M NaCl gradient (over a period of 8 hr) on a DEAE-Toyopearl column. Results of SDS-PAGE showed that Fraction II.1 included 2 major proteins of molecular weight 60 kD and 100 kD, Fraction II.2 included 3 major proteins of 35 kD, 60 kD, and 100 kD, and Fraction II.3 included 2 major proteins of 43 kD and 60 kD. Fraction II.3 demonstrated the highest CMCase activity (10 units/mg of protein) but showed low washing activity (using a sub-Micro Denim Wash test) and specific CMCase activity of 1 unit/mg. Fraction II.2 did not show any of the washing activity (but CMCase activity was 0.7 U/mg). Thus, Fractions II.1 and II.3 were purified further.

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Table 24. Properties of C1 Enzymes

	pH	25 kD endo	70(60) kD	70 kD endo	60 kD endo (II.1)	100kD (II.1)	43kD endo (II.3)	60kD endo (II.3)
5								
			CMCase Activity (U/mg)					
10	5.0	4.6	1.10	3.5	0.70	0.03	1.02	1.32
	6.0	5.0	0.83	3.8	0.52	0	1.01	1.07
	7.0	3.9	0.65	3.0	0.45	0	0.90	1.03
15								
			RBB-CMCase (U/mg)					
	5.0	8.2	0.12	1.5	0.90	0	0.82	1.21
20	6.0	8.8	0.10	2.7	0.84	0	0.75	1.14
	7.0	6.6	0.07	2.4	0.68	0	0.73	1.12
25								
			FPA (U/mg)					
	5.0	1.0	0.17	0.61	0.31	0	0.45	0.52
30								
			Endoglucanase (viscometric) (U/mg)					
	5.0	2.26	0.50	2.8	0.21	0	0.15	0.27
35								
			Avicelase (U/mg)					
	5.0	0.16	0.31	0.18	0.03	0	0.01	0.01
40								
			β -Glucosidase (U/mg)					
	5.0	0	0.02	0.16	0.02	0	0.02	0
45								

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Table 24 - cont'd

β -Glucanase (U/mg)								
5.0	0.66	0.07	2.4	1.3	0	3.9	4.4	
Xylanase (U/mg)								
5.0	0.40	0.16	0.50	0.06	0.01	0.07	0.01	
Micro Denim Wash Activity								
5.0	+++	-	++	n.d.	n.d.	n.d.	n.d.	
7.0	+++	-	++	n.d.	n.d.	n.d.	n.d.	
Sub-Micro Denim Wash Activity								
5.0	n.d.	n.d.	n.d.	+++	-	+	+	
7.0	n.d.	n.d.	n.d.	+++	-	+++	++	
n.d. = not determined								

11. Sub-Micro Denim Wash Test. This test was performed on fragments of real Indigo-stained denim in 2 mL of buffered enzyme solution (2 units CMCase activity) at 50°C for 2 hours in the conditions of excessive mechanical stress. The level of cellulase performance was evaluated by panel score according to the color reduction after the samples were dried.

12. Purification of Fraction II.1. Fraction II.1 was applied to a Macro prep Q column equilibrated with 0.03 M acetate buffer, pH 4.75, and the adsorbed proteins were eluted in a NaCl gradient (0 - 0.3 M). Two protein peaks were obtained but only the first one showed CMCase activity. SDS-PAGE of the material from the first peak showed that proteins with 60 kD and 100 kD were isolated in a homogeneous state. According to IEF data, the 60 kD and 100 kD

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proteins possessed an acidic pI of about 3. The activities of the 60 kD and 100 kD proteins toward different substrates are shown in Table 24. The 60 kD protein designated as 60 kD(II.1)-endoC1 was found to possess endoglucanase, CMCase, RBB-CMCase, FPA and β -glucanase activities at pH 5 (0.2, 0.7, 0.9, 0.3, and 1.3 units/mg of protein, respectively, as shown in Table 24). Avicelase, β -glucosidase, and xylanase activities were rather low. This combination of activities shows that the 60kD(II.1)-endoC1 is an endoglucanase. The 60kD(II.1)-endoC1 also possesses high washing activity (by the Sub-Micro Denim Wash test) both at pH 5 and at pH 7 (per Table 24). The pH dependence of CMCase and RBB-CMCase activities for this protein showed maxima at pH 4.0 - 4.5, with 50% of maximal activity toward CMC and 85% of maximal activity toward RBB-CMC retained at pH 6, and 15-20% of both activities retained at pH 9 and 10 (see Table 25).

The 100 kD protein from Fraction II.1 was designated as 100kD(II.1) protein and almost did not have cellulase activity (Table 24). This protein possessed only very low CMCase (0.03 U/mg) and xylanase (0.008 U/mg) activities and could not be determined to be a cellulytic enzyme. According to the Sub-Micro Denim Wash test, the 100 kD (II.1) protein did not demonstrate any stonewashing activity (Table 24) and also failed to show any protease activity at either pH 5 or pH 7.

13. Purification of Fraction II.3. Fraction II.3 was also purified by Macro Prep Q chromatography. The adsorbed proteins were eluted in 0.2 - 0.6 M NaCl gradient (the start buffer was 0.03 M acetate, pH 4.75). SDS-PAGE of the obtained fractions after the Macro Prep Q chromatography showed that the 43 kD and 60 kD proteins were obtained in homogeneous form. Isoelectrofocusing of these fractions showed that both the 43 kD and 60 kD proteins had pI values of about 3. The 43 kD and 60 kD proteins were designated 43kD(II.3)-endoC1 and 60kD(II.3)-endoC1, respectively. The activities of these enzymes toward different substrates (see Table 24) showed that they had similar specific CMCase, FPA, avicelase, and xylanase activities. The 60kD(II.3)-endoC1 possessed higher specific

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RBB-CMCase, endoglucanase and FPA activities (Table 24). At the same time it should be stressed that the 43kD(II.3)-endoC1 and 60kD(II.3)-endoC1 possessed very little stonewashing activities at pH 5 (using the Sub-Micro Denim Wash test). However, both 43kD(II.3)-endoC1 and 60kD(II.3)-endoC1 demonstrated
5 remarkable stonewashing activity at pH 7, and at the same time the 43kD(II.3)-endoC1 had higher stonewashing activity compared to the 60kD(II.3)-endoC1. As seen from the pH dependencies in Table 25, 43kD(II.3)-endoC1 showed a broad pH optimum (from pH 4.5 to 8) in the case of both CMCase and RBB-CMCase activities. The 43kD(II.3)-endoC1 possessed 50% CMCase and 70% RBB-CMCase
10 activities from a maximum at pH 9 and 20% of both CMCase and RBB-CMCase at pH 10. In contrast, 60kD(II.3)-endoC1 had a narrow pH optimum at pH 4 - 4.5 toward CMC and a broad pH optimum (from pH 4 to 8) toward RBB-CMC and 30% RBB-CMCase activity being retained at pH 9.

It should be noted that in all cases of purified proteins disclosed herein,
15 molecular weights were determined using gel electrophoresis (especially SDS-PAGE) and reference proteins of known molecular weight as standards. As with all analyses using such methods, the results are only approximate and some variation in molecular weight may be observed as different gels are run by different workers using different sets of molecular weight standards as references.

20 Such purified, and partially purified, enzyme preparations are highly useful as components of detergent, fabric softening, depilling, color brightening and stone washing compositions. Thus, the above isolated and purified enzyme preparations find utility in such applications according to the present invention. Thus, methods for stone washing, fabric softening, depilling, color brightening and cleansing as
25 heretofore recited herein, as well as typical methods for accomplishing such applications as already disclosed in the literature will readily employ such purified, or partially purified, enzyme preparations, and compositions containing such, as main or additive agents in effecting the goals of such procedures. The use of other and different purified, or partially purified, enzyme preparations in such
30 applications is known in the literature with many enzymes in commercial use.

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Table 25. The Effect of pH on CMCase and RBB-CMCase activities of C1
Cellulase Enzymes

CMCase Activity, 50°C (%)			
pH	60kD(II.1)-endo	43kD(II.3)-endo	60kD(II.3)-endo
3.5	85	80	85
4.0	100	100	100
5.0	65	100	85
6.0	50	100	70
7.0	45	90	65
8.0	25	70	45
9.0	17	30	30
10.0	15	20	10
11.0	15	5	5
RBB-CMCase activity, 40°C (%)			
pH	60kD(II.1)-endo	43kD(II.3)-endo	60kD(II.3)-endo
3.5	85	85	80
4.0	100	100	100
5.0	90	100	100
6.0	85	95	95
7.0	70	90	90
8.0	50	80	85
9.0	20	70	70
10.0	15	20	30
11.0	15	15	15

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It should be understood that the neutral and/or alkaline cellulases heretofore described can have different enzymatic activities depending on the chemical structure of the substrate used in measuring the activity and the particular assay method employed to measure activity. Thus, the purified, or partially purified, neutral and/or alkaline cellulases will show different pH/activity profiles depending on the assay method and substrates employed. To resolve any confusion as to the nature of the activities and properties of the substantially purified cellulase enzymes prepared by the methods of this example, the following is a description of the activities and properties measured for the cellulases of the purified fractions.

The purified, or partially purified, cellulases prepared herein showed both endoglucanase and/or cellobiohydrolase activity when the appropriate substrate was employed. Thus, the cellulase preparations that showed endoglucanase activities all had pI values between about 3 and about 4.5. More specifically, these fractions included cellulases (endoglucanases) having molecular weights and pI values as follows: MW about 25 kD (pI about 4.0), MW about 70 kD (pI about 4.2), MW about 60 kD (pI about 3.0) and MW about 43 kD (pI about 3.1). These cellulase fractions also contained proteins showing a cellobiohydrolase activity. More specifically, the latter had a MW of about 60 kD and pI about 4.2.

Methods of using the compositions and purified enzymes according to the present invention have been well disclosed in the literature, including many patents, whose disclosures are hereby incorporated by reference. These would include Clarkson (U.S. Patent 5,290,474), which discloses use of cellulase enzymes and cellulase enzyme-containing compositions, including surfactants and other additives, for use in aqueous wash media, detergent compositions, media designed to enhance color retention and/or restoration, as well as imparting improved softening and feel properties, especially to cotton-containing fabrics. The cellulase enzymes and cellulase-containing compositions according to the present invention are also intended for use in the same applications, specific descriptions of which are described in many, if not all, of the references cited. In addition, the utility of cellulase enzymes and compositions for applications such as harshness reduction, or

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660990-06039
5 fabric softening, is also taught in Barbesgaard et al (U.S. Patent 4,435,307), specifically disclosing the use of fungal cellulases, but not those of the genus *Chrysosporium*, at alkaline pH ranges and including various additive agents, such as those employed in conjunction with the novel cellulase and cellulase
10 compositions of the present invention, for harshness reduction, or fabric softening, and washing as a single operation. The cellulases and cellulase compositions of the present invention are similarly useful and the teachings of Barbesgaard with respect to such applications is specifically incorporated herein. In addition, the use of cellulase enzymes and cellulase compositions, other than the novel cellulases and cellulase compositions of the present invention, for applications to color
15 brightening are specifically disclosed in Boegh (European Patent EP 0 220 016), which teaching is specifically incorporated herein.

Of course, the novel cellulase enzymes and cellulase compositions of the present invention will be understood by those of skill in the art to be highly useful
20 for the same applications as disclosed in in the foregoing references, thus rendering the elucidation of any further details of such applications unnecessary. However, such purified, or partially purified, enzymes and enzyme-containing compositions are also useful in such applications as deinking and biobleaching of paper or pulp materials and method of doing so will readily suggest themselves to those of skill
25 in the art, especially after they review the teachings herein.

Example 13 -- C-1 Cellulase Production in 60 Liter Batch Fermentor

1. Inoculum Preparation

Inoculum preparations or starter cultures for the batch fermentation were prepared as follows. One milliliter (1 ml) of C-1 spore culture was used to inoculate each of two flasks to generate a total of 2.0 liters of inoculum. The
30 starter culture was incubated at 150 rpm, at 30°C for 56 hours.

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Medium for Inoculum Preparation*

	K ₂ HPO ₄	0.5	g/L
	MgSO ₄ • 7H ₂ O	0.15	
	KCl	0.05	
5	FeSO ₄ • 7H ₂ O	0.007	
	yeast extract (ohly KAT)	1.0	
	peptone (Hormel PSR 5)	10.0	
	lactose	10.0	
	glucose	10.0	
10	*The pH of the inoculum medium was adjusted to pH 7.0 with NaOH, the media was then autoclaved for 35 minutes at 121°C in two six liter baffled flasks each containing one liter of medium.		

2. Cellulase Production in 60 Liter Batch Fermenter
(Preparation of 47.0325)

The two liter shaker flask inoculum culture prepared above, was used inoculate 40 liters of medium contained in a 60 liter fermenter. The medium for fermentation was as follows:

Fermentation Medium*

	K ₂ HPO ₄	0.22	g/L
25	KH ₂ PO ₄	0.08	g/L
	(NH ₄) ₂ SO ₄	4.0	g/L
	Na ₃ citrate • 2H ₂ O	4.0	g/L
	MgSO ₄ • 7H ₂ O	0.03	g/L
	CaCl ₂ • 2H ₂ O	0.4	g/L
30	FeSO ₄ • 7H ₂ O	0.5	mg/L
	MnSO ₄ • 7H ₂ O	0.5	mg/L
	ZnSO ₄ • 7H ₂ O	0.2	mg/L
	CoCl ₂ • 6H ₂ O	0.24	mg/L
	lactose	5.0	g/L
35	yeast extract (ohly KAT)	0.05	g/L
	defatted cotton seed flour (Pharmamedia)	5.0	g/L
	cellulose (Signmacell 50)	20.0	g/L
	pH	7.0	

40 *The 40 liters medium was in deionized water, and was sterilized for 45 minutes at 121°C.

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After inoculation of the fermentation medium, pH was maintained above 6.9 by addition of NH_3 and below 7.1 by addition of H_2SO_4 . The fermenter was incubated for 64 hours with agitation and aeration as necessary to maintain dissolved oxygen greater than 30% of saturation.

3. Recovery of Cellulase Activity

Suspended solids from the fermented culture were removed by filtration on large Buchner funnel using Whatman 54 filter paper and 10 g/L Celite 503 as filter aid. The filtrate was collected, and the cellulase concentrated by ultrafiltration using 10,000 MW cutoff hollow fiber filter. The concentrate was freeze dried. The dried concentrate was designated cellulase preparation 47.0325. The activity of this preparation is given in Table 4.

Example 14 -- Mutation procedure used to generate mutant stain of C-1

A spore suspension was prepared using a Pridham agar plate (4 g/L yeast extract, 10 g/L malt extract, 10 g/L glucose, 15 g/L agar) containing a sporulated culture of strain C1. The plate was flooded with 10 ml of 0.05% Tween 80. The suspension was transferred to a sterile screw cap tube and vortexed on high for 1 minute. The suspension was then filtered through a column to remove mycelium. Spores were counted and diluted to 7×10^5 spores per ml in water. Ten mls of the spore suspension were transferred to a standard glass petri dish. The spores were irradiated for 75 seconds at $720 \mu\text{Watts}/\text{cm}^2$ using a Pen-Ray UV bulb. The spore suspension was gently stirred throughout the irradiation using a sterile paper clip as a magnetic stir bar. Following irradiation, the spore suspension was taken to a foil wrapped tube, diluted in water and plated in dim light to NH_4 minimal medium as defined below. After incubating 20 days at 30 degrees C, a colony was identified as a large colony with a large zone of cellulose clearing around the colony.

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NH₄ Minimal Medium, pH 7.51 g/L K₂HPO₄

0.1g/L KCl

0.3g/L MgSO₄•7H₂O

5 0.1g/L NaCl

16mg/L FeCl₃•6H₂O1.92g/L(NH₄)₂SO₄

15g/L Difco Noble agar

2.5g/L acid swollen cellulose (added as a 1.25% stock after autoclaving)

10 0.5g/L sodium deoxycholate (added after autoclaving)

Example 15 -- C-1 Mutant Cellulase Production in 60 Liter Batch Fermentation Flasks (Preparation Of 47.0528)15 1. Inoculum Preparation

Preparations of starter cultures for the fed batch fermentation were prepared as described in Example 13 (section 1).

20 2. Cellulase Production In 60 Liter Batch Fermentation

The two liter inoculum was used to inoculate 40 liters of fermentation medium as described below.

Fermentation Medium*

25	K ₂ HPO ₄	0.44	g/L
	KH ₂ PO ₄	0.16	g/L
	(NH ₄) ₂ SO ₄	3.0	g/L
	Na ₂ citrate •2H ₂ O	4.0	g/L
	MgSO ₄ •7H ₂ O	0.06	g/L
	CaCl ₂ •2H ₂ O	0.8	g/L
30	FeSO ₄ •7H ₂ O	0.1	mg/L
	MnSO ₄ •7H ₂ O	0.04	mg/L
	ZnSO ₄ •7H ₂ O	0.04	mg/L
	CoSO ₄ •6H ₂ O	0.048	mg/L
	lactose	5.0	g/L
35	yeast extract (ohly KAT)	0.1	g/L

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defatted cotton seed flour (Pharmamedia)	10.0	g/L
cellulose (Sigmacell 50)	20.0	g/L

*The 40 liters medium was in deionized water, and sterilized by autoclaving for 45 minutes at 121°C.

3. Fermentation Conditions

The pH was maintained at around 7.0 and controlled by addition of NH_3 at pH above 6.9, and addition of H_2SO_4 at pH below 7.1. Incubation time was 87 hours, agitation and aeration were as necessary to maintain dissolved oxygen greater than 30% of saturation. At 40 hours, 3.0 liters of feed solution as described below, was added at a rate of 5.0 ml each 5 minutes.

Feed Solution for Fermenter

K_2HPO_4	0.88	g/L
KH_2PO_4	0.32	g/L
$(\text{NH}_4)_2\text{SO}_4$	4.0	g/L
$\text{Na}_2\text{citrate} \cdot 2\text{H}_2\text{O}$	4.0	g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.12	g/L
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.16	g/L
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.2	mg/L
$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	0.08	mg/L
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.08	mg/L
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.096	mg/L
lactose	20.0	g/L
yeast extract (ohly KAT)	0.2	g/L
Pharmamedia	20.0	g/L
cellulose (Sigmacell 50)	20.0	g/L

4. Recovery of Cellulase Activity

Suspended solids were removed by filtration on large Buchner funnel using Whatman 54 filter paper and 10 g/L Celite 503 as filter aid. The filtrate was collected and cellulase concentrated by ultrafiltration using 10,000 MW cutoff hollow fiber filter. The concentrate was dried by freeze-drying. The concentrate was designated cellulase preparation 47.0528 (activity is given in Table 4).

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Example 16 - Assay of Cellulase Activity Using Cellazyme Assay

This assay was carried out using Cellazyme C tablets and the assay kit obtained from Megazyme (Aust) Pty. Ltd., Sydney, NSW 2101, Australia. The substrate used is Azurine-crosslinked HE-celulose (AZCL-Cellulose) supplied commercially as cellazyme C tablets, ready for use. Briefly, 0.5 mL aliquots of enzyme preparation (diluted if necessary) in 0.025 M acetate buffer (pH 4.5) are equilibrated to 40°C for 5 minutes in glass test tubes (16 X 122 mm). The test reaction is then initiated by addition of a Cellazyme C tablet (without stirring). After exactly 10 minutes at 40°C, the reaction is terminated by addition of Trizma Base solution (10.0 mL, 2% w/v, from Sigma Chemical Co., St. Louis, MO) and then vortexing. The tubes are then allowed to stand for about 5 minutes at room temperature whereupon the slurry is stirred again, filtered through a Whatman No. 1 (9 cm) filter circle and absorbance of the filtrate measured at 590 nm. The absorbance measurements are read against a blank containing both substrate and enzyme but prepared by adding the Trizma Base to the enzyme solution prior to addition of the cellazyme C tablet. This slurry is left at room temperature rather than 40°C. A single blank is used for each set of determinations and was used to zero the spectrophotometer.

In this assay, one unit of enzyme activity is defined as the amount of enzyme required to release one micromole of glucose reducing sugar equivalents per minute under the defined assay conditions. Endo-cellulase activity was then determined by reference to a standard curve (a sample curve was supplied with the kit but could be readily generated in the laboratory, if different particular enzyme, enzyme dilution and conditions are to be employed for a given set of experiments).

Using this assay for our own neutral/alkaline enzyme activity, we found endo-Cellulase activity (taking the activity at pH 7 as 100%) to be 92.4% (at pH 6), 75.6% (at pH 5.0) and 69.7% (at pH 4.0).

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Example 17 - Detergent Wash Test for De-pilling and Color Brightening (Anti-fading)

Tests were carried out according to the AATCC monograph, "Standardization of Home Laundry Test Conditions" in the AATCC Technical Manual, 1997 (revised 1995), using a regular Kenmore home top-loading washer, using a Kenmore home tumble-dryer. Three different styles of red adult-size socks (88% cotton, 10% polyester, 2% lycra) were used as test garments (plain ribbed knit, heavy ribbed knit and waffle weave). The socks were divided into 3 groups with an equal number of each style in each group, with a non-washed garment of each style used as a reference. The approximate weight of each group was 1 kg.

The samples of detergent were prepared prior to each wash as follows: a) Cheer Triple Guard (as purchased in the U.S.A. and commonly at alkaline pH) as is (to demonstrate the de-pilling and color care properties of cellulases originally present in Cheer, b) thermally treating the sample of Cheer to inactivate all of the enzymes originally present in the detergent (to limit the performance of Cheer to its components other than enzymes. For thermal inactivation, a 40 g sample of Cheer was suspended in 200 mL of water and heated on high (1000 W) in a home microwave for 5 minutes, the final temperature reached being 95°C. Then, repeating the same steps (a) and (b) above, but adding 5 g of C1 to the preparation.

The wash/dry cycles were performed 25 times for each group. Each group was washed throughout the 25 wash cycles using 1 kg of garments to 20 liters of wash liquor (water level in the washer was low) and 40 g of only one of the above mentioned detergent preparations. The temperature was set on hot-hot, the wash duration was 30 minutes, followed by regular, high speed centrifugation. The dryer temperature was set on high and the drying cycle lasted 45 minutes. The testing was complete after 25 washes, when the garments were evaluated, by several different groups/panels skilled in the art, for de-pilling and color brightening.

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A summary of the procedure is as follows:

Preparation(s)	Inactivation of original Cheer enzymes	Addition of Enzymes from AARL
a. Cheer (complete)	No	No
b. Cheer (no enzymes)	Yes	No
c. Cheer + C1	Yes	Yes (5 g of C1)

The results of the rating for each wash group were as follows (where all 3 styles of socks were rated the same):

	Cheer (complete)	Cheer (enzymes inactivated)	Cheer + C1
De-pilling	++++	-	+++
Color Brightening	++	-	++++

It should be understood that the examples and embodiments described herein are intended for illustrative purposes only and that various modifications or changes in light thereof will readily suggest themselves to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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Claims

1. An isolated culture of *Chrysosporium lucknowense* Garg 27K having accession number VKM F-3500D.

2. A composition having neutral and/or alkaline cellulase activity, obtained by a method which comprises growing a wild type or mutant fungus of the genus *Chrysosporium* in culture in a suitable medium, wherein the fungus is *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, or *Chrysosporium tropicum*.

3. A composition according to claim 2 wherein the fungus is *Chrysosporium lucknowense*.

4. A composition having neutral and/or alkaline cellulase activity, obtained by a method which comprises growing a mutant fungus of the genus *Chrysosporium* in culture in a suitable medium.

5. A composition according to claim 2 wherein the fungus is *Chrysosporium lucknowense* Garg 27K, accession number VKM F-3500D.

6. A composition according to claim 2 wherein the fungus is a mutant strain of *Chrysosporium lucknowense* Garg 27K.

7. A composition according to any one of claims 2-6 having optimal cellulase activity at a temperature from about 40°C to about 60°C, at a pH from about 5.0 to about 12.0.

8. A composition according to any one of claims 2-6 having at least 50% of the optimal cellulase activity, at a pH from about 6.0 to about 7.0, at a temperature from about 40°C to about 60°C.

5 9. A composition according to any one of claims 2-6 wherein said cellulase activity is assayed by any one of the CMCase, RBBCMCase, endovisco-metric or filter paper activity assays.

10 10. A substantially purified and isolated protein fraction, obtained from a composition according to claim 2 or claim 4, and having at least 50% of its maximal cellulase activity at a pH between about 6.0 and about 7.0 as measured by any one of the CMCase, RBBCMCase, endovisco-metric or filter paper activity assays.

15 11. An endoglucanase obtained from a fraction according to claim 10, having a molecular weight of about 25 kD and pI of about 4.

20 12. An endoglucanase obtained from a fraction according to claim 10, having a molecular weight of about 70 kD and a pI of about 4.

 13. An endoglucanase obtained from a fraction according to claim 10, having a molecular weight of about 60 kD and a pI of about 3.

25 14. An endoglucanase obtained from a fraction according to claim 10, having a molecular weight of about 43 kD and a pI of about 3.

 15. A cellobiohydrolase obtained from a fraction according to claim 10, having a molecular weight of about 60 kD and a pI of about 4.

16. A substantially purified and isolated neutral and/or alkaline cellulase enzyme, isolated from a protein fraction according to claim 10, and having a pI of between about 3 and about 5.5.

5 17. A cellulase according to claim 16 wherein said cellulase possesses either endoglucanase or cellobiohydrolase activity.

10 18. A cellulase according to claim 16 wherein said cellulase retains at least 50% of its maximal cellulase activity at a pH between about 6.0 and about 7.0.

19. An endoglucanase obtained from a fraction according to claim 10 and having a molecular weight of about 25 kD.

15 20. An endoglucanase obtained from a fraction according to claim 10 and having a molecular weight of about 70 kD.

20 21. An endoglucanase obtained from a fraction according to claim 10 and having a molecular weight of about 43 kD.

22. A detergent composition containing one or more purified enzymes isolated from a protein fraction according to claim 10, and further comprising a surfactant.

25 23. A fabric softening composition containing one or more purified enzymes obtained from the protein fraction according to claim 10.

30 24. A composition for the enzymatic treatment of cellulosic fibers or cellulosic fabrics, comprising a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus

Chrysosporium, said composition having a pH between about 8.0 and about 12.0.

25. The composition of claim 24, wherein the fungus is selected from the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium pruinsum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, and *Chrysosporium tropicum*.

26. The composition of claim 25, wherein the fungus is *Chrysosporium lucknowense*.

27. A composition according to any one of claims 24-26, wherein the cellulase is isolated or obtained from a wild-type or mutant fungus of the genus *Chrysosporium*.

28. A composition according to any one of claims 24-26, further comprising one or more components selected from the group consisting of pumice stones, abrasives, softeners, solvents, preservatives, bleaching agents, bluing agents, fluorescent dyes, antioxidants, solubilizers, detergents, surfactants, enzymes, builders, anti-redeposition agents, buffers, caking inhibitors, masking agents for factors inhibiting the cellulase activity, and cellulase activators.

29. The composition of claim 28, wherein the cellulase is isolated or obtained from a wild-type or mutant fungus of the genus *Chrysosporium*.

30. The composition of claim 24, wherein the pH is between 10.0 and about 11.0.

31. The composition of claim 25, wherein the pH is between 10.0 and about 11.0.

5 32. The composition of claim 26, wherein the pH is between 10.0 and about 11.0.

33. The composition of claim 27, wherein the pH is between 10.0 and about 11.0.

10 34. The composition of claim 28, wherein the pH is between 10.0 and about 11.0.

15 35. The composition of claim 29, wherein the pH is between 10.0 and about 11.0.

20 36. A composition for the enzymatic treatment of cellulosic fibers or cellulosic fabrics, comprising a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus *Chrysosporium*, said composition further comprising one or more components selected from the group consisting of proteinases, detergents, and surfactants.

25 37. The composition of claim 36, wherein the fungus is selected from the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium pruinsum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, and *Chrysosporium tropicum*.

30 38. The composition of claim 37, wherein the fungus is *Chrysosporium lucknowense*.

39. A composition as described in any one of claims 36-38, wherein the cellulase is isolated or obtained from a wild-type or mutant fungus of the genus *Chrysosporium*.

5 40. A composition for the enzymatic treatment of cellulosic fibers or cellulosic fabrics, having at least 124 units of endo-1,4- β -glucanase activity per gram of dry composition, as measured by an endoviscometric assay, of a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus *Chrysosporium*.

10 41. The composition of claim 40, wherein the fungus is selected from the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium pruinsum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, and *Chrysosporium tropicum*.

15 42. The composition of claim 41, wherein the fungus is *Chrysosporium lucknowense*.

20 43. A composition for the enzymatic treatment of cellulosic fibers or cellulosic fabrics, having at least 124 units of endo-1,4- β -glucanase activity per gram of dry composition, as measured by an endoviscometric assay, of a cellulase isolated or obtained from a wild-type or mutant fungus of the genus *Chrysosporium*.

25 44. The composition of claim 43, wherein the fungus is selected from the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium pruinsum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, and *Chrysosporium tropicum*.

30

45. The composition of claim 44, wherein the fungus is *Chrysosporium lucknowense*.

46. A composition for the enzymatic treatment of cellulosic fibers or cellulosic fabrics, having at least 191 units of endo-1,4- β -glucanase activity per gram of dry composition, as measured by an endoviscometric assay, of a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus *Chrysosporium*.

47. The composition of claim 46, wherein the fungus is selected from the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium pruinsum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, and *Chrysosporium tropicum*.

48. The composition of claim 47, wherein the fungus is *Chrysosporium lucknowense*.

49. A composition for the enzymatic treatment of cellulosic fibers or cellulosic fabrics, having at least 191 units of endo-1,4- β -glucanase activity per gram of dry composition, as measured by an endoviscometric assay, of a cellulase isolated or obtained from a wild-type or mutant fungus of the genus *Chrysosporium*.

50. The composition of claim 49, wherein the fungus is selected from the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium pruinsum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, and *Chrysosporium tropicum*.

51. The composition of claim 50, wherein the fungus is *Chrysosporium lucknowense*.

52. A composition for the enzymatic treatment of cellulosic fibers or
cellulosic fabrics, having at least about 964 units of endo-1,4- β -glucanase
activity per gram of dry composition, as measured by an endovisco-metric assay,
of a cellulase whose amino acid sequence is encoded by a nucleic acid sequence
from a wild-type or mutant fungus of the genus *Chrysosporium*.

53. The composition of claim 52, wherein the fungus is selected from
the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*,
Chrysosporium pruinsum, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*,
Chrysosporium merdarium, *Chrysosporium queenslandicum*, and
Chrysosporium tropicum.

54. The composition of claim 54, wherein the fungus is *Chrysosporium lucknowense*.

55. A composition for the enzymatic treatment of cellulosic fibers or
cellulosic fabrics, having at least about 964 units of endo-1,4- β -glucanase
activity per gram of dry composition, as measured by an endovisco-metric assay,
of a cellulase isolated or obtained from a wild-type or mutant fungus of the
genus *Chrysosporium*.

56. The composition of claim 55, wherein the fungus is selected from
the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*,
Chrysosporium pruinsum, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*,
Chrysosporium merdarium, *Chrysosporium queenslandicum*, and
Chrysosporium tropicum.

57. The composition of claim 57, wherein the fungus is *Chrysosporium lucknowense*.

58. A laundry detergent composition, comprising a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus *Chrysosporium*, further comprising one or more surfactants.

59. The composition of claim 58, wherein the fungus is selected from the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium pruinsum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, and *Chrysosporium tropicum*.

60. The composition of claim 59, wherein the fungus is *Chrysosporium lucknowense*.

61. A laundry detergent composition, comprising a cellulase isolated or obtained from a wild-type or mutant fungus of the genus *Chrysosporium*, further comprising one or more surfactants.

62. The composition of claim 61, wherein the fungus is selected from the group consisting of *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium pruinsum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, and *Chrysosporium tropicum*.

63. The composition of claim 62, wherein the fungus is *Chrysosporium lucknowense*.

64. A cellulase composition having cellulase activity at neutral and/or alkaline pH, obtained from a mutant or wild-type fungus of the genus *Chrysosporium*.

5 65. A cellulase composition according to claim 64, wherein the fungus is of the species *Chrysosporium lucknowense*.

66. A cellulase composition according to claim 65, wherein the fungus is *Chrysosporium lucknowense* Garg 27K, accession number VKM F-3500D.

10 67. A method for producing a composition having neutral and/or alkaline cellulase activity, said method comprising growing a wild type or mutant fungus of the genus *Chrysosporium* in culture in a suitable medium.

15 68. The method, according to claim 67, wherein the fungus is *Chrysosporium lucknowense*, *Chrysosporium pannorum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium merdarium*, *Chrysosporium queenslandicum*, or *Chrysosporium tropicum*.

20 69. The method, according to claim 68, wherein the fungus is *Chrysosporium lucknowense*.

70. The method according to claim 69, wherein the fungus is *Chrysosporium lucknowense* Garg 27K, accession number VKM F-3500-D.

25 71. The method according to claim 67, wherein the fungus is a mutant strain of the genus *Chrysosporium*.

30 72. The method according to claim 71 wherein the fungus is a mutant strain of *Chrysosporium lucknowense* Garg 27K.

73. A method of stonewashing denim fabric or denim jeans, said method comprising treating said denim fabric or denim jeans with a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus *Chrysosporium*.

5

74. A method of biopolishing, defribillating, bleaching, dyeing, or desizing textiles comprising treating said textiles with a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus *Chrysosporium*.

10

75. A method of deinking or biobleaching paper or pulp, said method comprising treating said paper or pulp with a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus *Chrysosporium*.

15

76. A method for enhancing the softness or feel of cellulose or cotton-containing fabric, comprising treating said fabric with a cellulase whose amino acid sequence is encoded by a nucleic acid sequence from a wild-type or mutant fungus of the genus *Chrysosporium*.

20

77. A method according to any one of claims 73-76, wherein the cellulase is isolated or obtained from a wild-type or mutant fungus of the genus *Chrysosporium*.

25

78. A method according to any one of claims 73-76, wherein the fungus is *Chrysosporium lucknowense*.

79. A method according to claim 77, wherein the fungus is *Chrysosporium lucknowense*.

30

80. A method for generating mutant strains of the genus *Chrysosporium* which produce enhanced cellulase activity at neutral and/or alkaline pH's, comprising

- (a) mutating spores of a fungus of the genus *Chrysosporium*;
- (b) culturing the spores from step (a); and
- (c) screening the cultures from step (b) for enhanced levels of neutral and/or alkaline cellulase activity.

81. The method of claim 80 wherein the mutating step comprises exposing the spores to ultraviolet light or a chemical mutagen.

82. The method of claim 81 wherein the chemical mutagen is nitrous acid, N-methyl-N'-nitro-N-nitrosoguanidine, or 4-nitroquinolone-N-oxide.

83. A mutant strain of the genus *Chrysosporium* obtained by the method of any one of claims 80-82.

84. A method of isolating genes encoding cellulase enzymes of *Chrysosporium* comprising:

- a) isolating a protein from a neutral and/or alkaline cellulase composition produced by a wild type or mutant *Chrysosporium*;
- b) sequencing all or part of the protein isolated in step (a);
- c) producing a nucleic acid probe derived from the sequence of step (b);
- d) screening a wild type or mutant *Chrysosporium* library with the nucleic acid probe of step (c);
- e) isolating a nucleic acid sequence recognized by the probe; and
- f) sequencing the nucleic acid sequence isolated in step (e).

85. A nucleic acid sequence obtained by the method of claim 84.

86. An isolated nucleic acid whose sequence encodes a cellulase enzyme, wherein the cellulase enzyme is isolated or obtained from a wild-type or mutant fungus of the genus *Chrysosporium*.

5 87. An isolated nucleic acid whose sequence encodes a cellulase according to any one of claims 11-21.

88. A recombinant expression vector comprising a nucleic acid sequence according to any one of claims 85-87.

10 89. A host cell containing a recombinant expression vector of claim 88.

90. A host cell according to claim 89 wherein the host cell is selected from the group consisting of yeast cells, fungal cells, plant cells, and bacterial cells.

15 91. A host cell according to claim 90 wherein the host cell is a fungal cell selected from the group consisting of *Trichoderma*, *Aspergillus*, *Humicola*, *Penicillium*, *Chrysosporium*, and *Neurospora*.

20 92. A method of culturing a fungus of the genus *Chrysosporium*, wherein the fungus is grown in a medium containing inorganic salts, carbon sources, and organic nitrogen sources, at a pH between about 5 and 8.

25 93. A method of culturing a fungus of the genus *Chrysosporium* according to claim 92, wherein the pH is between about 6.5 and 7.5.

94. A method of culturing a fungus of the genus *Chrysosporium* according to claim 92, wherein the pH is between about 6.9 and 7.1.

COMBINED DECLARATION AND POWER OF ATTORNEY FOR
ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL
DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART APPLICATION

As a below name inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CHRYOSPORIUM CELLULASE AND METHODS OF USE

the specification of which

a. ☐ is attached hereto

b. ☒ was filed on April 8, 1999 as application Serial No. 09/284,152 and was amended on _____ (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

c. ☒ was described and claimed in International Application No. PCT/US97/17669 filed on 30 September, 1997 and as amended on _____ (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby specify the following as the correspondence address to which all communications about this application are to be directed:

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☒ I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or under § 365(b) of any foreign application(s) for patent or inventor's certificate or under § 365(a) of any PCT international application(s) designating at least one country other than the U.S. listed below and also have identified below such foreign application(s) for patent or inventor's certificate or such PCT international application(s) filed by me on the same subject matter having a filing date within twelve (12) months before that of the application on which priority is claimed:

☐ The attached 35 U.S.C. § 119 claim for priority for the application(s) listed below forms a part of this declaration.

<u>Country/PCT</u>	<u>Application Number</u>	<u>Date of filing (day, month, yr)</u>	<u>Date of Issue (day, month, yr)</u>	<u>Priority Claimed</u>
				[] YES [] NO
				[] YES [] NO
				[] YES [] NO

[] I hereby claim the benefit under 35 U.S.C. § 119(e) of any U.S. provisional application(s) listed below.

Provisional Application No.

Date of Filing (day, month, yr)

ADDITIONAL STATEMENTS FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART OR PCT INTERNATIONAL APPLICATION(S) (DESIGNATING THE U.S.)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or under § 365(c) of any PCT international application(s) designating the U.S. listed below.

08/731,170	October 10, 1996	Patented
US/PCT Application Serial No.	Filing Date	Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)
US/PCT Application Serial No.	Filing Date	Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)

[X] In this continuation-in-part application, insofar as the subject matter of any of the claims of this application is not disclosed in the above listed prior United States or PCT international application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or Imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and/or agents with full power of substitution and revocation, to prosecute this application, to receive the patent, and to transact all business in the Patent and Trademark Office connected therewith: John A. Diaz (Reg. No. 19,550), John C. Vassil (Reg. No. 19,098), Alfred P. Ewert (Reg. No. 19,887),

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_____ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

-3-

David H. Pfeffer (Reg. No. 19,825), Harry C. Marcus (Reg. No. 22,390), Robert E. Paulson (Reg. No. 21,046), Stephen R. Smith (Reg. No. 22,615), Kurt E. Richter (Reg. No. 24,052), J. Robert Dailey (Reg. No. 27,434), Eugene Moroz (Reg. No. 25,237), John F. Sweeney (Reg. No. 27,471), Arnold I. Rady (Reg. No. 26,601), Christopher A. Hughes (Reg. No. 26,914), William S. Feiler (Reg. No. 26,728), Joseph A. Calvaruso (Reg. No. 28,287), James W. Gould (Reg. No. 28,859), Richard C. Komson (Reg. No. 27,913), Israel Blum (Reg. No. 26,710), Bartholomew Verdirame (Reg. No. 28,483), Maria C.H. Lin (Reg. No. 29,323), Joseph A. DeGirolamo (Reg. No. 28,595), Michael P. Dougherty (Reg. No. 32,730), Seth J. Atlas (Reg. No. 32,454), Andrew M. Riddles (Reg. No. 31,657), Bruce D. DeRenzi (Reg. No. 33,676), Michael M. Murray (Reg. No. 32,537), Mark J. Abate (Reg. No. 32,527), Alfred L. Haffner, Jr. (Reg. No. 18,919), Harold Haidt (Reg. No. 17,509), John T. Gallagher (Reg. No. 35,516), Steven F. Meyer (Reg. No. 35,613) and Kenneth H. Sonnenfeld (Reg. No. 33,285) of Morgan & Finnegan, L.L.P. whose address is: 345 Park Avenue, New York, New York, 10154; and Edward A. Pennington (Reg. No. 32,588), Michael S. Marcus (Reg. No. 31,727) and John E. Hoel (Reg. No. 26,279) of Morgan & Finnegan, L.L.P., whose address is 1775 Eye Street, Suite 400, Washington, D.C. 20006.

[] I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from _____

_____ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

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[] I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from _____ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

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400

Table 1. Demographic characteristics of the study population	
Age (years)	Mean (SD)
Male	55.2 (10.5)
Female	56.8 (11.2)
Marital status	
Married	78.5%
Single	21.5%
Education level	
High school or above	65.2%
Below high school	34.8%
Occupation	
Professional	12.3%
Managerial	18.7%
Technical	25.4%
Service	32.1%
Unemployed	11.5%
Health status	
Good	72.3%
Fair	27.7%
Chronic diseases	
Hypertension	45.6%
Diabetes	32.1%
Heart disease	28.9%
Stroke	15.4%
Arthritis	38.7%
Chronic kidney disease	12.8%
Chronic lung disease	9.5%
Chronic liver disease	7.2%
Chronic mental illness	4.1%
Chronic pain	18.3%
Chronic fatigue	11.7%
Chronic insomnia	14.5%
Chronic depression	8.9%
Chronic anxiety	6.3%
Chronic stress	10.2%
Chronic anger	7.8%
Chronic sadness	9.1%
Chronic loneliness	11.4%
Chronic isolation	8.7%
Chronic social withdrawal	10.5%
Chronic lack of interest	9.8%
Chronic loss of appetite	11.2%
Chronic weight loss	10.1%
Chronic fatigue	11.7%
Chronic weakness	10.3%
Chronic dizziness	9.6%
Chronic headache	10.8%
Chronic back pain	11.5%
Chronic joint pain	12.1%
Chronic muscle pain	11.9%
Chronic skin problems	10.4%
Chronic hair loss	9.7%
Chronic nail problems	10.2%
Chronic eye problems	11.1%
Chronic ear problems	10.6%
Chronic nose problems	10.9%
Chronic throat problems	11.3%
Chronic mouth problems	11.6%
Chronic genital problems	11.8%
Chronic urinary problems	12.0%
Chronic bowel problems	12.2%
Chronic skin problems	12.4%
Chronic hair loss	12.6%
Chronic nail problems	12.8%
Chronic eye problems	13.0%
Chronic ear problems	13.2%
Chronic nose problems	13.4%
Chronic throat problems	13.6%
Chronic mouth problems	13.8%
Chronic genital problems	14.0%
Chronic urinary problems	14.2%
Chronic bowel problems	14.4%
Chronic skin problems	14.6%
Chronic hair loss	14.8%
Chronic nail problems	15.0%
Chronic eye problems	15.2%
Chronic ear problems	15.4%
Chronic nose problems	15.6%
Chronic throat problems	15.8%
Chronic mouth problems	16.0%
Chronic genital problems	16.2%
Chronic urinary problems	16.4%
Chronic bowel problems	16.6%
Chronic skin problems	16.8%
Chronic hair loss	17.0%
Chronic nail problems	17.2%
Chronic eye problems	17.4%
Chronic ear problems	17.6%
Chronic nose problems	17.8%
Chronic throat problems	18.0%
Chronic mouth problems	18.2%
Chronic genital problems	18.4%
Chronic urinary problems	18.6%
Chronic bowel problems	18.8%
Chronic skin problems	19.0%
Chronic hair loss	19.2%
Chronic nail problems	19.4%
Chronic eye problems	19.6%
Chronic ear problems	19.8%
Chronic nose problems	20.0%
Chronic throat problems	20.2%
Chronic mouth problems	20.4%
Chronic genital problems	20.6%
Chronic urinary problems	20.8%
Chronic bowel problems	21.0%
Chronic skin problems	21.2%
Chronic hair loss	21.4%
Chronic nail problems	21.6%
Chronic eye problems	21.8%
Chronic ear problems	22.0%
Chronic nose problems	22.2%
Chronic throat problems	22.4%
Chronic mouth problems	22.6%
Chronic genital problems	22.8%
Chronic urinary problems	23.0%
Chronic bowel problems	23.2%
Chronic skin problems	23.4%
Chronic hair loss	23.6%
Chronic nail problems	23.8%
Chronic eye problems	24.0%
Chronic ear problems	24.2%
Chronic nose problems	24.4%
Chronic throat problems	24.6%
Chronic mouth problems	24.8%
Chronic genital problems	25.0%
Chronic urinary problems	25.2%
Chronic bowel problems	25.4%
Chronic skin problems	25.6%
Chronic hair loss	25.8%
Chronic nail problems	26.0%
Chronic eye problems	26.2%
Chronic ear problems	26.4%
Chronic nose problems	26.6%
Chronic throat problems	26.8%
Chronic mouth problems	27.0%
Chronic genital problems	27.2%
Chronic urinary problems	27.4%
Chronic bowel problems	27.6%
Chronic skin problems	27.8%
Chronic hair loss	28.0%
Chronic nail problems	28.2%
Chronic eye problems	28.4%
Chronic ear problems	28.6%
Chronic nose problems	28.8%
Chronic throat problems	29.0%
Chronic mouth problems	29.2%
Chronic genital problems	29.4%
Chronic urinary problems	29.6%
Chronic bowel problems	29.8%
Chronic skin problems	30.0%
Chronic hair loss	30.2%
Chronic nail problems	30.4%
Chronic eye problems	30.6%
Chronic ear problems	30.8%
Chronic nose problems</	

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1. Review the declaration and verify the correctness of all information therein; and
2. Review the specification and the claims, including any amendments made to the claims.

FORM: ADDED-PG.DEC
Rev. 05/26/98

ADDED PAGE TO COMBINED DECLARATION
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Before signing this declaration, each person signing must:

1. Review the declaration and verify the correctness of all information therein; and
2. Review the specification and the claims, including any amendments made to the claims.

After the declaration is signed, the specification and claims are not to be altered.

ADDED PAGE TO COMBINED DECLARATION
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1. Review the declaration and verify the correctness of all information therein; and
2. Review the specification and the claims, including any amendments made to the claims.

After the declaration is signed, the specification and claims are not to be altered.

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ADDED PAGE TO COMBINED DECLARATION
AND POWER OF ATTORNEY FOR
SIGNATURE BY THIRD AND SUBSEQUENT INVENTOR

Full name of seventh joint inventor, if any Philip T. OLSON

Inventor's signature* Phillip T Olson 5/21/99
date

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* Before signing this declaration, each person signing must:

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Full name of eighth joint inventor, if any Arkady Panteleimonovich SINITSYN

Inventor's signature* A. Sinitsyn 5/19/99
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[X] ATTACHED IS/ARE ADDED PAGE(S) TO COMBINED DECLARATION AND POWER OF ATTORNEY FORM FOR SIGNATURE BY THIRD AND SUBSEQUENT INVENTORS

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After the declaration is signed, the specification and claims are not to be altered.

To the inventor(s):

The following are cited in or pertinent to the declaration attached to the accompanying application:

Title 37, Code of Federal Regulation, § 1.56

Duty to disclose information material to patentability.

(a) A patent by its very nature is affect with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

Title 35, U.S. Code § 101

Inventions patentable

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Title 35 U.S. Code § 102

Conditions for patentability; novelty and loss of right to patent

A person shall be entitled to a patent unless –

- (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent,
- (b) the invention was patented or described in a printed publication in this or foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States, or
- (c) he has abandoned the invention, or
- (d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate filed more than twelve months before the filing of the application in the United States, or
- (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or
- (f) he did not himself invent the subject matter sought to be patented, or
- (g) before the applicant's invention thereof the invention was made in this country by another had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other ...

Title 35, U.S. Code § 103

Conditions for patentability; non-obvious subject matter

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

Title 35, U.S. Code § 112 (in part)

Specification

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise and exact terms also enable any person skilled in the art to which it pertains, or with which it is mostly nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Title 35, U.S. Code § 119

Benefit of earlier filing date in foreign country; right of priority

An application for patent for an invention filed in this country by any person who has, or whose legal representatives or assigns have, previously regularly filed an application for a patent for the same invention in a foreign country which affords similar privileges in the case of applications filed in the United States or to citizens of the United States, shall have the same effect as the same application would have if filed in this country on the date on which the application for patent for the same invention was first filed in such foreign country, if the application in this country is filed within twelve months from the earliest date on which such foreign application was filed; but no patent shall be granted on any application for patent for an invention which had been patented or described in a printed publication in any country more than one year before the date of the actual filing of the application in this country, or which had been in public use or on sale in this country more than one year prior to such filing.

Title 35, U.S. Code § 120

Benefit or earlier filing date in the United States

An application for patent for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in an application previously filed in the United States, or as provided by section 363 of this title, which is filed by an inventor or inventors named in the previously filed application shall have the same effect, as to such invention, as though filed on the date of the prior application, if filed before the patenting or abandonment of or termination of proceedings on the first application or an application similarly entitled to the benefit of the filing date of the first application and if it contains or is amended to contain a specific reference to the earlier filed application.

Please read carefully before signing the Declaration attached to the accompanying Application.

If you have any questions, please contact Morgan & Finnegan, L.L.P.

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Rev. 5/21/98